CRANFIELD UNIVERSITY

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Symbiotic nitrogen fixation enhancement due to magnetite nanoparticles

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This thesis is submitted in partial fulfilment of the requirements for the degree of Doctoral of Bio-nanotechnology

Abstract

Population pressure on food production motivates the search for new ways to increase the productivity of arable land, especially land rendered marginal by salinity or aridity. The global thesis motivating this work is that nanotechnology can benefit agriculture. My specific thesis is that that part of nanotechnology concerned with nanoparticle production can benefit soybean yield. I have focused on symbiotic nitrogen fixation, and systematically investigated the effects thereon of magnetite nanoparticles introduced into the rhizosphere.My main finding is that the presence of these nanoparticles increases nodulation -- both the number of nodules and the size of individual nodules. Since the experiments were carried out on plants provided with minimal nutrients, there was no corresponding increase in vegetative growth. Some evidence was obtained for the nanoparticles enhancing the "molecular dialogue" between soybean root and the Brady*rhizobia* that become incorporated in the nodules. A secondary finding is that the nanoparticles enhance the growth rate of Brady*rhizobia* in culture, which is advantageous for the preparation of inocula. Furthermore, coating soybean seeds with nanoparticles and Brady*rhizobia* prior to planting enhances survival of the bacteria, and therefore increases the efficiency of subsequent nodulation.

Summary

My thesis is that nanotechnology can be powerfully used to enhance processes in agriculture. My object is the soybean plant, chosen because of its global agricultural importance. A particular challenge to which I wished to contribute is how to enhance the ability of the soybean plant to flourish under adverse conditions. In particular, I have investigated whether magnetite nanoparticles and magnetite nanobiocomposites affect *Bradyrhizobium* growth rate (BGR) in liquid media, *Bradyrhizobium* viability on the soybean seeds, with a view to controlling the sensitivity of the bacteria to high pH, salinity, desiccation and, hence, survival in extreme conditions, and symbiotic nitrogen fixation. Treatments comprised different concentrations of magnetite nanoparticles or magnetite nanobiocomposites suspended in liquid medium (yeast mannitol broth, YMB) mixed with and adhering to *Bradyrhizobium japonicum* bacteria and adjusted to different pH and salinities in the individual experiments. The most important measured variables were growth rate coefficient (GRC), mean generation time (MGT), number of generations (NG) of bacteria before the stationary phase; the number of viable cells (N_v), the number of viable *Bradyrhizobium* cells as inocula on the seeds (N_l), water activity (a_w), oxidation reaction potential (ORP), quantity of secreted lipochitooligosaccharide (LCO) from bacterial cells, the quantity of genistein secreted from the soybean root, dry leaf weight, dry weight stem, dry root weight, dry nodule weight, number of nodules, number of nodes on the stem, number of branches, and plant height. Experimental results were obtained in eight groups of experiments. The results can be summarized as follows:

1-The nanoparticles increased GRC and NG and decreased MGT; the optimal quantity of nanoparticles was determined. In the presence of the nanoparticles the pH of the culture remained almost constant from zero time to 96 hours of growth, but without nanoparticles the pH increased significantly, from 6.8 to 7.1 during the same interval.

2-Nanoparticles under conditions of alkaline and acidic pH could increase GRC and NG, and decrease MGT. Application of the nanoparticles could preserve oxygen availability such that the ORP and the a_w were changed beneficially under extreme conditions. Interaction effects of pH and nanoparticles on the bacterial growth could enhance NG and GRC and decrease MGT, however, if the concentration of magnetite nanoparticles was increased beyond a certain value there was an inverse effect on the physiological growth indices.

3-Added nanoparticles at high salt concentrations could preserve the population of *Bradyrhizobium* cells and could increase NG and GRC and decrease MGT. The effect of salinity stress on the BGR decreased in the presence of the magnetite nanoparticles.

4-The nanoparticles increased the viability of *Bradyrhizobium japonicum* residing on the soybean seeds, at low temperature (4 °C) by about 50% during about 5 days.

5-The nanoparticles induced secretion of the LCO from *Bradyrhizobium japonicum*.

6-The decoration of *Bradyrhizobium* cells by nanoparticles induced secretion of genistein from soybean roots.

7-The nanoparticles increased the number of nodules, the total weight of nodules, and the quantity of fixed nitrogen.

8-Magnetite nanobiocomposites increased nodule number, total weight of nodules, the amount of generated C_2H_4 (as an indicator of nitrogen fixation)

Bradyrhizobium growth indices (in culture) were affected by magnetite nanoparticles, because they have a pH buffering effect in liquid medium, presumably achieved either by adsorbing alkaline bacterial secretions or by reacting with them. The literature suggests nanoparticles may complex and inactivate oxygen scavengers in the medium, and also catalyse reactions tending to keep the pH neutral.

The results illustrated that oxygen reaction potential (ORP) increased during *Bradyrhizobium* growth without nanoparticles, likely due to releasing reactive oxygen species (ROS) and reactive nitrogen species (RNS) in the medium, whereas in the presence of the magnetite nanoparticles the secretions of bacterial cells were presumably sequestered by the nanoparticles, hence the nanoparticles diminished the ORP, because the nanoparticles are expected to be moderate reducing reagents under low or high pH stress and regulate water activity. The nanoparticles could catalytically convert superoxides to peroxides and oxygen molecules and then produce water molecules, also these nanoparticles are enable to absorb water molecules temporarily and then they may be prevented from decreasing available oxygen during bacterial culture.

In fact these nanoparticles have the capacity of preserving oxygen molecules by neutralization of the ROS and RNS. Preserving optimum conditions like the a_w and the ORP at extreme pH could enhance N_f, NG and GRC, and decrease MGT. However, results indicated that a high concentration of magnetite nanoparticles has inverse effects on the optimum physiological indices of *Bradyrhizobium* growth, possibly because a high density of nanoparticles increases osmotic pressure and/or redox potential in the liquid media are unbalanced.

Acidity and alkaline stress decreased *Bradyrhizobium* growth, while in the presence of magnetite nanoparticles the bacterial cells had considerably enhanced growth and survival. The nanoparticles are involved in the synthesis of some complex proteins, which are able to control transporter proteins in the membrane and decrease the impact of direct ion toxicity effects. Acidity stress significantly reduces lipopolysaccharide (LPS)--fatty acid complexes in the

bacterial cell membrane and decreased cell viability, but in the presence of the nanoparticles bacterial cells could withstand the low pH stress in the medium. Besides, alkaline stress decreases available iron for the bacterial cell, consequently synthesis of metalloproteins like cytochromes, which are necessary for oxidation, during respiration of the bacterial cells, is limited, hence also limiting many different cellular processes needing energy, such as biosynthesis, and active transport. Magnetite nanoparticles compensate iron deficiency in bacterial cells.

The *Bradyrhizobium japanicum* is salt-sensitive. Cell viability decreased due to salinity stress in the medium, while application of the magnetite nanoparticles decreased the salinity effect on the viability. Likewise, the salinity stress was unable to constrain bioavailable iron for *rhizobial* cells in the medium. The literature suggests that under salinity stress and growth-limiting conditions the general metabolism of *rhizobial* cells declines. After salinity shocks the cells accumulate and synthesise and/or take up compatible solutes that are osmoprotective, like glycogen and trehalose. But this response depends on the level of salinity stress; even changing cell morphology, dimension and polysaccharide modification were unable to decrease the effect of salinity stress. Magnetite nanoparticles on the cell wall could diminish the impacts of salinity stress. In addition, preservation of the cell wall by the nanoparticles likely leading to the protection of cell membrane choline, which is a precursor to the synthesis of some osmoprotectants like glycine and betain. Although a high density of nanoparticles in the medium was unable to diminish effectively *Bradyrhizobium* growth, and this response likely is due to activation of the ferric uptake repressor (Fur) protein. The literature suggests that a high concentration of iron in the medium causes repression of the expression of genes for siderophore biosynthesis and transport, and this repression is mediated by the Fur protein.

Nodulation and symbiotic nitrogen fixation is due to molecular dialogue between symbiotic partners (*rhizobia* and host plants). Initially secreted flavonoids from host plant roots induce the bacterial cell to secrete the LCO as

a nodulation factor (nodf). Generally infection of microsymbionts into root hair cells will lead to activation of a defense mechanism, because of which a positive signal between flavonoids and nodf trigger symbiosis between microsymbionts and the host root. In fact the nodf prevents the invasion of secreted molecules that causes the death of microsymbionts. Therefore, the ability of the nodf to persist in extreme conditions is a main factor in nodulation and symbiotic nitrogen fixation under environmental stress.

Introducing an inducer agent for the nodf could be a way to increase the possibility of successful nodulation and symbiotic nitrogen fixation under environmental stress. Experiments showed that 40 and 60 $\mu g\ ml^{-1}$ of magnetite nanoparticles with 10–20 nm diameter favour the symbiotic nitrogen fixation. Secreted genistein as an isoflavonoid of the soybean root, and the nodf of *Bradyrhizobium japanicum* were affected compared with the nanoparticle-free system.

Furthermore, the fabrication of magnetite nanoparticles with polymers such as methylcellulose, soylecithin, and polyvinylalcohol (PVA) — i.e., nanobiocomposite-- increased cell viability; this type of fabricated nanoparticles has an enhanced capacity to scavenge radical molecules in medium and protect the cell wall. In addition they are able to act as effective carriers for the bacterial cells on seeds, protecting them against environmental stress like desiccation.

The yields of the vegetative components of the soybean were not affected by application of magnetite nanoparticles and nanobiocomposites, however, some of the components' responses was contradiction.

Among these components an increasing number of nodules and their weight were the most important factors leading to increase the quantity of fixing nitrogen, because increasing the number of nodules implies an increased area of bacteroids for fixing nitrogen and producing ammonium ion. Therefore the results indicate that, likely, the free nanoparticles and bacteria coated with the

nanoparticles could effectively act as fertilizer and promote the vegetative growth indices of soybean.

Hence, these magnetite nanoparticles can both enhance the culture of *Bradyrhizobium japanicum* (prior to inoculating the seeds) and enhance growth of the plant. Since these results were obtained in culture media, the effects and responses need to be tested in soil under agricultural conditions.

Keywords:

Soybean, *Bradyrhizobium*, environmental stress,

bio-nanotechnology.

Glossary

AD	Antioxidant defence
ASH-GSH	Ascorbate- glutathione
ATP	Adenosine triphosphate
ATR	Acid tolerance response
BGR	*Bradyrhizobium* growth rate
B. japonicum	*Bradyrhizobium japonicum*
bv	biovar
D	Desiccation
EC	Electrical conductivity
EPS	Exopolysaccharides
ESP	Exchangeable Sodium Percentage
FAO	Food and agriculture organization
FTIR	Fourier transform infrared
Fur	Ferric uptake repressor
GlcNAc	N-acetylglucosamine
GPA	Glucose peptone agar
GRC	Growth rate coefficient
HMN	Hybrid magnetite nanoparticles
HPLC	High Pressure liquid chromatography

IT	Infection thread
Lb	Leghemoglobin
LCO_s	Lipochitooligosaccharides
LCO	Lipochitooligosaccharide
LPS	Lipopolysaccharides
MGT	Mean generation time
MoFe	Molybdenum-iron protein
NG	Number of generations
Nod Bj-V ($C_{18:1}$ MeFuc)	Nodule factor from *Bradyrhizobium japonicum*
nodABCIJ	Nodulation genes
nodf	Nodulation factor
N_P	Magnetite nanoparticle
ORP	Oxidation reaction potential
PVA	Polyvinyl alcohol
PVC	Polyvinyl chloride
RBD	Randomized blocking design
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RUPB	Ribulose biphosphate synthesis
RWC	Relative water content
SG	Seed and glass beads

SOV	Sources of variation
SNF	Symbiotic nitrogen fixation
SOD	Super oxide dismutases
T	Treatments
TEM	Transmission electron microscopy
Temp.	Temperature
tRNA	Transfer ribonucleic acid
XRD	X-ray diffraction
YMB	Yeast mannitol broth
YMA	Yeast mannitol agar

PUBLICATIONS

1. In journals:

A) Ghalamboran, M. R., Ramsden, J. J., Ansari F. Growth rate enhancement of *Bradyrhizobium japonicum* due to magnetite nanoparticles. J. Bionanosci. (2009); 3, 1-6.

2. Published in the proceedings:

B) Ghalamboran, M. R., Ramsden, J. J. Magnetite nanoparticles enhance growing rate of *Bradyrhizobium japonicum*. Proceedings of the Nanotech Conference, (2009) May 3-7; Houston, USA.

C) Ghalamboran, M.R., Ramsden, J.J. Viability of *Bradyrhizobium japonicum* on the soybean seeds due to magnetite nanoparticles. Proceedings of the International Conference on Biotechnology and Food Engineering, (2010) March 28-31; Rio de Janeiro, Brazil.

D) Ghalamboran, M. R., Ramsden, J. J. Magnetite nanoparticles as moderators of extreme conditions during *Bradyrhizobium japonicum* growth. Proceedings of the Nanotech, Cleantech, Microtech Conferences, (2010) June 21-25; Anaheim, CA, USA.

3. In preparation:

a) Effect of physical characteristics of Fe_3O_4 nanobiocomposites on *Bradyrhizobium japonicum* growth.
Authors: M.R. Ghalamboran, Matthew Kershaw, Kazem Khavazi, Jeremy J. Ramsden.

b) Response of Nod factor (nodf) excretion of *Bradyrhizobium japonicum* to the presence of magnetite nanoparticles.
Authors: M.R. Ghalamboran, Jeremy J. Ramsden.

c) Response of genistein excretion as bioflavonoid from soybean roots due to coated *Bradyrhizobium japonicum* by magnetite nanoparticles.
   Authors: M.R. Ghalamboran, Jeremy J. Ramsden.

d) Magnetite nanoparticles enhance signal molecule production between *Bradyrhizobium* and soybean roots.
   Authors: M.R. Ghalamboran, Jeremy J. Ramsden.

e) Symbiotic nitrogen fixation enhancement of soybean plant due to using magnetite nanoparticles.
   Authors: M.R. Ghalamboran, Jeremy J. Ramsden.

f) The functional role of magnetite nanoparticles in liquid media of *Bradyrhizobium japonicum*.
   Authors: M.R. Ghalamboran, Jeremy J. Ramsden.

This thesis is dedicated to Imam Zaman (peace be upon him) and Prophet Jesus (peace be upon him) that they will surely come for saving humanity from darkness and ignorances.

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1 Introduction

1.1 Background

1.1.1 The prospects for agriculture in the future

Agriculture is a need for human survival and humanity can be proud of himself for inventing means and processes to facilitate production of food and clothes. Agricultural development depends on soil, water, plant and climate. Growth and development in agriculture is accompanied by population growth and increased demands for food. Food and Agriculture Organization (FAO) reports show that in recent years the growth rates of world agricultural production and crop yields have slowed, whereas the population of world is expected to grow from 6.9 billion in 2010 to 9.1 billion in 2050, an increase of 24% (Figure 1-1) [66,202]. There is a serious fear that the world may not be able to grow enough food and other commodities to ensure that future populations are adequately fed.

Since1990 to 2007 arable land area has increased by 4.2 percent in the world (Figure 1-2), while population growth has been 20%. Therefore the share of arable land per person (from 0.24% to 0.20%) has diminished 20%. As a result there would have been shortage of food if no other solution had been sought [64,65].

The main reasons for slow growth of arable land are natural disasters and incompatible government policies [16,94]. In addition conventional methods for increasing land use efficiency have caused other environmental problems, which led to more natural disaster. One of these conventional methods is to use artificial fertilizers to compensate nutrients deficiency in soils (Figure 1-3). These methods have also disturbed the balancing and biological cycle of nutrients in nature [71].

Although since 1990 to present to agricultural lands was added about 4%, these new lands are low quality and have serious problems in soil fertility [63,65].

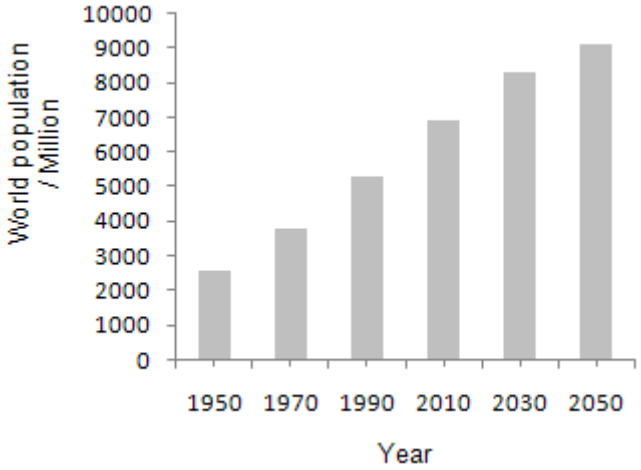


Figure 1-1. Trend of growth of world population [66].

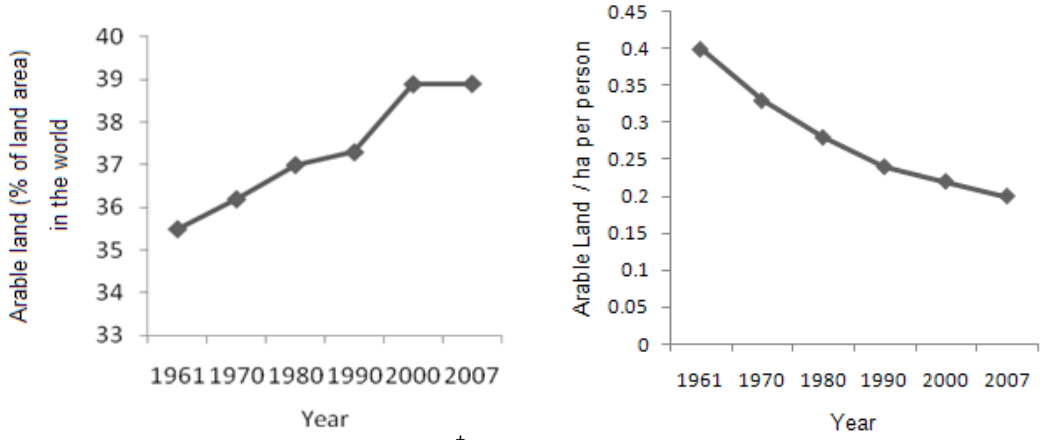


Figure 1-2. Trends growth of arable land† area in the world, and share of arable land per person [65].

† Arable land includes land defined by the FAO as land under temporary crops (double-cropped areas are counted once), temporary meadows for mowing or for pasture, land under market or kitchen gardens, and land temporarily fallow. Land abandoned as a result of shifting cultivation is excluded.

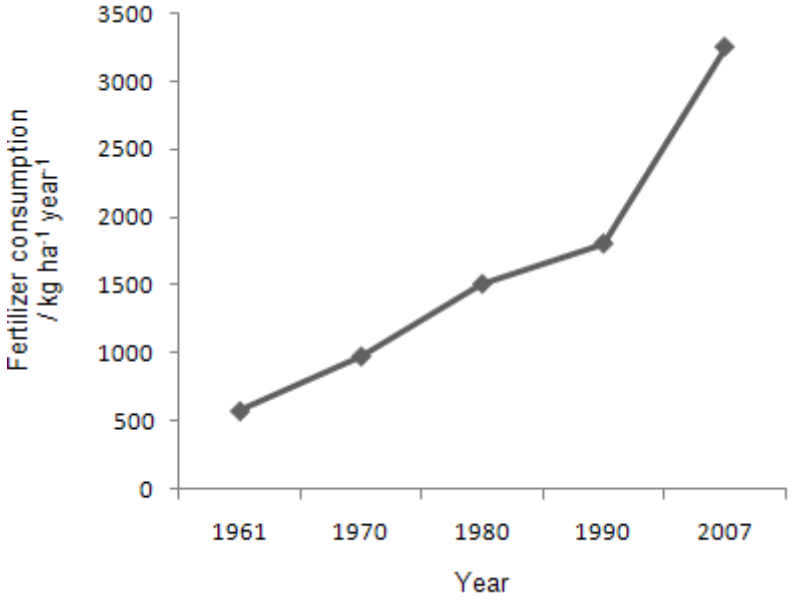


Figure 1-3. Trend of fertilizers† consumption in the world [63, 64].

1.1.2 The economical importance of soybean

Soybean is the most important plant source of protein in the world. 33% of food protein worldwide is derived from legumes [87]. Soybean seed contained 40% of protein and 20% oil, so that predominately soybean and peanut provide 35% of vegetable oil in the world [75,87,190]. Likewise cultivation and producing kinds of soybean products is easy and cheaper than other industrial plants. Basically, the reasons for the development of soybean return to its multi-purpose uses: food, feed, fuel, milk and industrial materials such as paint, inks, and plastics [158].

By 2009 Masuda and Goldsmith that reported that out of 183.9 million tons of world supply/demand of soybean in 2001-2003, about 10% of its products were directly consumed as food (5.9) or feed (3.8) but 84.2% of

† Fertilizers cover nitrogenous, potash, and phosphate (including ground rock phosphate). Traditional nutrients--animal and plant manures--are not included.

soybean was crushed into soy-oil and soy-meal [158]. Soy-oil is processed to vegetable oil for human consumption, and recently used as a biodiesel feedstock. Soy-meal is used as feed for livestock, aquaculture [158].

Soybean seed with about 40% protein is one of the fondamental sources of plant protein for the human, and as a legume seed, in company with vegetables may contribute between 15-25% of the dietary protein intake [186, 75].

Among all existing crops, Soybean only uses 0.9% of total of nitrogenous fertilizers in the world (Figure 1-4). Furthermore soybean as a legume could contribute N_2 to other plants [186]. Thus soybean can promote yield of other plants in intercropping or rotations. Previous researches showed that it is often assumed that a portion of the nitrogen fixed by intercropped legume is made available to the associated non-legume during the growing season [186]. Direct transfer ways of nitrogen from legumes to other plants are decaying roots and nodules of legumes, although this transfer may not occur under all conditions, or might only occur slowly with time [186]. However if the benefits of crop legumes in intercropping or rotations cannot be explained solely in terms of residual fixed nitrogen, but based on other researches extra yield from a rotation can result from improvement in soil structure following legumes, or improvements in soil water-holding and buffering capacity, and increased nutrient availability associated with incorporation of legume residual, promotion of soil microbial activity and possibly heterotrophitic nitrogen fixation following addition of legume residues [186,187,42]

Development of soybean is implies lower consumption of at least 50-60% of nitrogen fertilizer compared with other crops as a result produces less than 50% of pollutants in air and soils. The harvest area in the recent two decades has slightly increased for soybean (Figure 1-5). Also in 2007 the world totally produced up to 219 million tons soybean (grain). The greatest soybean producing countries were the USA with up to 72 million tons, Brazil with up to 57 million tons, India with 109 million tons and Argentina with 47 million tons [158].

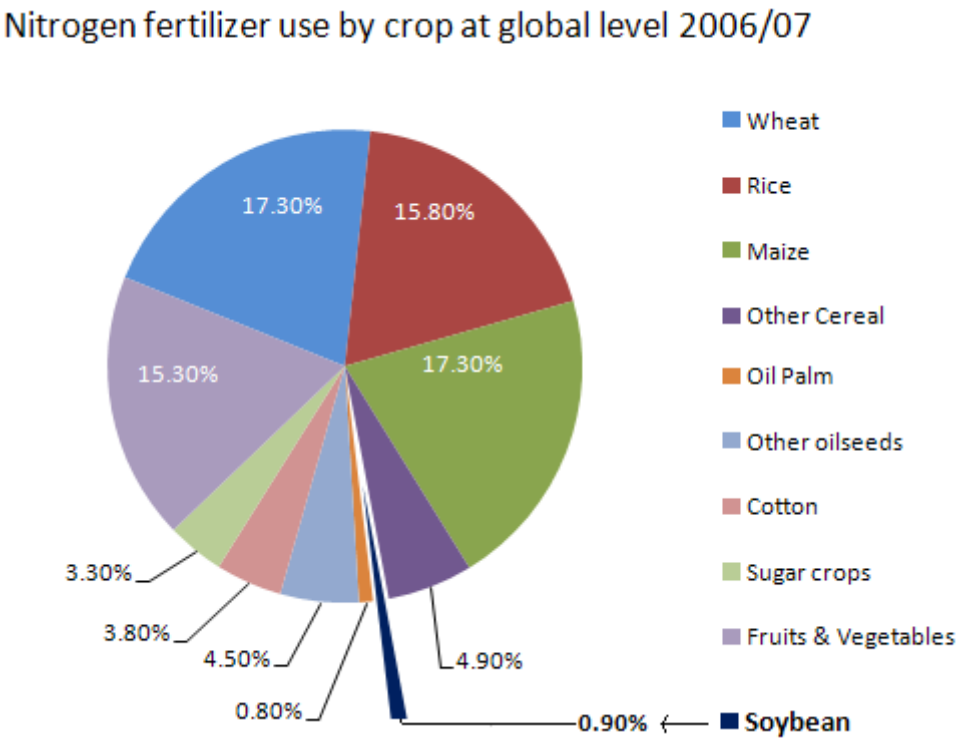


Figure 1-4. Consumption of nitrogen fertilizer by crops [99,63].

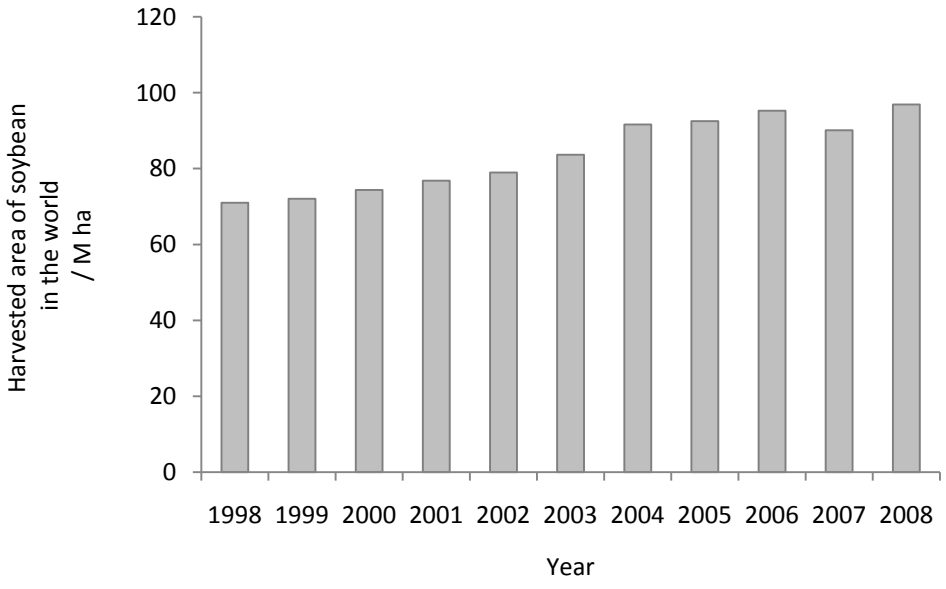


Figure 1-5. Area harvested of soybean in the world [67].

1.1.3 Challenges of developing soybean

Generally challenges return to lack of appropriate varieties, planting date, density of plants, irrigation, nutrients management and agro–technical problems. One of the specific challenges is an inactive symbiotic relation of *Bradyrhizobium japonicum* with root cells due to environmental stress effects and nitrogenous fertilizers consumption. In order to achieve high yield potential, soybean must sustain high photosynthesis rates and accumulate amounts of nitrogen in seeds. On average, 50 to 60% of soybean nitrogen demand was met by biological nitrogen fixation. Although the amount of nitrogen fixed was not sufficient to replace nitrogen fertilizer from the field. Antagonism between nitrate concentration in the soil solution and the symbiotic nitrogen fixation process in the nodules is the main constraint the legumes faces in terms of increasing nitrogen uptake [234] when no other abiotic stress that reduce symbiotic nitrogen fixation activity occurs such as soil moisture stress [61,43], soil pH [199] or soil temperature [61] Due to increasing aridity and salinity of arable lands (desertification) [148,267,268], average symbiotic nitrogen fixation efficiency is decreasing [258,259,268], therefore any gaps between soybean nitrogen demand and nitrogen supply by symbiotic nitrogen fixation must be met by nitrogen uptake from other sources. Thus farmers prefer use nitrogen fertilizer, because of that the economical development of soybean in comparison with other crops will not be justified.

Despite its problems, the consumption of nitrogenous fertilizers is increasing (Tables 1-1 and 2), and this affects of environmental stress such as pH, salinity, water deficit, and toxicants, affecting in turn the symbiotic activity and bacteria. Moreover, maximum of soybean yield depends on symbiotic nitrogen fixing efficiency and the symbiotic relation depends on bacterial cells viability and secreted signal molecules (isoflavonoids) from root cells, and viability depends on environmental stress.

Table 1-1. World fertilizer consumption, 2007/8–2011/12 [63].

Percent annual growth fertilizers			
	N	**P**	**K**
World	1.4	2.0	2.4
Africa	2.9	1.0	2.0
North America	0.3	0.5	0.7
Latin America	2.4	2.8	2.9
West Asia	1.7	1	2.4
South Asia	2.2	3.5	4.2
East Asia	1.3	1.9	3.3
Central Europe	1.8	1.2	1.0
West Europe	- 0.3	- 0.7	0.0
East Europe & Central Asia	2.4	4.5	1.6
Oceania	4.9	1.7	2.1

Table 1-2. World nitrogen supply and demand balance, 2007/2008-2011/2012, [63]; the numbers are thousand tonnes.

	2007/2008	**2008/2009**	**2009/2010**	**2010/2011**	**2011/2012**
Total supply	131 106	136 252	140 732	147 748	154 199
Total demand	127 820	130 409	133 059	136 198	139 140
Surplus	3 286	5 843	7 673	11 550	15 059

1.1.4 Sources of nitrogen supplying of soybean

There are two sources of available nitrogen to the plant. First, the plant can take nitrogen from the soil, accounting up to 50% of the total nitrogen needed for growth. The soil is the first choice as a nitrogen source because this process requires less energy compared with the second nitrogen source. The remaining nitrogen comes from the well-known process called nitrogen fixation, a process carried out by plants in the legume family (such as soybean, clover, and alfalfa).

1.1.5 Botany of soybean

Soybean plant with scientific name *Glycine max*, a legume native to China, it is one of major sources of vegetable protein and oil for human and animal consumption, and for industrial usage. The valued portion of the plant is the seed, which contains about 40% protein and 21% oil [20,63,75,87]. Members of the family Leguminosae are consumed as dry mature seeds (grain legumes or pulses) or as immature green seeds in the pod. Legumes include beans, forage crops and oil seeds like the groundnut, lupin, and soybean, grown for their oil and protein. Soybean is classed as an oilseed. It is an annual plant. Its botanical taxonomy is given in Table 1-3.

Table 1-3. Taxonomy of soybean [48]

Kingdom	*Plantae*
Phylum	*Magnoliophyta*
Class	*Magnoliopsida*
Order	*Fabales*
Family	*Fabaceae*
Subfamily	*Faboideae*
Genus	*Glycine*
Species	*Glycine max*

The leaves are trifoliate. The fruit is a hairy pod that grows in clusters of 3–5, and usually containing 2–4 (rarely more) seeds (Figure 1-6).

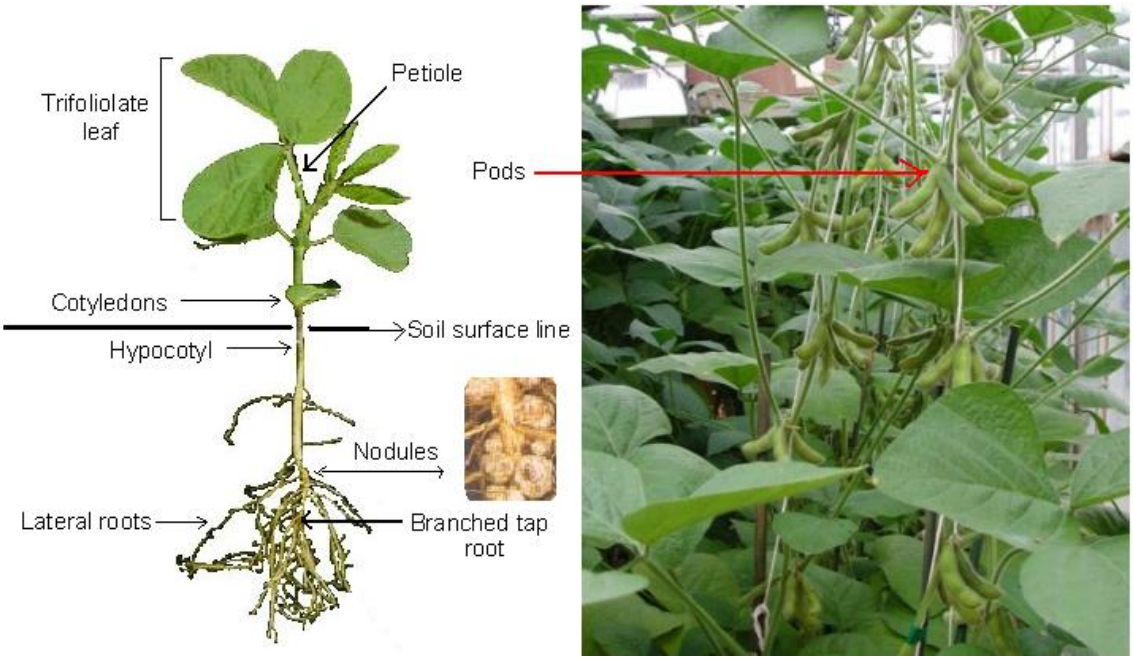


Figure 1-6. Botany of soybean plant.

1.1.6 Taxonomy of rhizobium

The *rhizobia* genus comes from the Latin language meaning “root living”. It is classified based on morphological and biochemical characteristics, genetic fingerprinting, fatty-acid methyl ester analysis, and 16S rRNA gene-sequence analysis [48,103].

These bacteria induce nodule formation in the root and stem of legumes. Currently the *rhizobia* consist of approximately 50 species in about 12 genera, some of which are listed in Table 1-4 [48].

Table 1-4. Genera and some species of the rhizobium [48]

Genera	***Species***	***Hosts***
Allorhizobium	*A. undicola*	*Neptunia natans, Acacia, Faidherbia, Lotus*
Azorhizobium	*A. caulinodans*	*Sesbania rostrata*
	A. doebereinerae	*Sesbania virgata*
Blastobacter	*A. denitrificans*	*Aeschynomene indica*
Bradyrhizobium	*B. canariense*	*Chamaecytisus, Lupinus*
	B. elkanii	*Glycine max*
	B. japonicum	*Glycine max*
	B. liaoningense	*Glycine max*
	B. yuanmingense	*Lespedeza, Medicago, Melilotus*
Burkholderia	*B. caribensis*	*Mimosa diplotricha, Mimosa pudica*
	B. cepacia	*Alysicarpus glumaceus*
	B. phymatum	*Machaerium lunatum, Mimosa*
	B. tuberum	*Aspalathus spp.*
Devosia	*D. neptuniae*	*Neptunia natans*
Ensifer	*E. adhaerens*	*(not reported)*
Mesorhizobium	*M. amorphae*	*Amorpha fruticosa*
	M. chacoense	*Prosopis alba*
	M. ciceri	*Cicer arietinum*
	M. huakuii	*Astragalus sinicus, Acacia*
	M. loti	*Lotus corniculatus*
	M. mediterraneum	*Cicer arietinum*
	M. plurifarium	*Acacia Senegal, Prosopis juriflora, Leucaena*
	M. septentrionale	*Astragalus adsurgens*
	M. temperatum	*Astragalus adsurgens*
	M. tianshanense	*Glycyrrhiza pallidifloria, Glycine, Caragana, Sophora*
Ralstonia (Cupriavidus)	*R. taiwanensis*	*Mimosa*
Rhizobium	*R. etli*	*Phaseolus vulgaris, Mimosa affinis*
	R. galegae	*Galega orientalis, G. officinalis*
	R. gallicum	*Leucaena, Onabrychis, Macroptilium, Phaseolus vulgaris*
	R. giardinii	*Macroptilium, Phaseolus vulgaris, Desmanthus, Leucaena*
	R. hainanense	*Desmanthus sinuatum, Vigan, Arachis, Centrosema, stylosanthes*
	R. huautlense	*Sesbania herbacea*
	R. indigofera	*Indigofera*
	R. leguminosarum	
	bv *trifolii*	*Trifolium,*
	bv *viciae*	*Lathyrus, Lens, Pisum, Vicia,*
	bv *phaseoli*	*Phaseolus vulgaris*
	R. loessense	*Astragalus, Lespedeza*
	R. mongolense	*Medicago ruthenica, Phaseolus vulgaris*
	R. sullae	*Hedysarum coronilla,*
	R. tropici	*Phaseolus vulgaris, Dalea, Macroptilium, Leucaena, Onabrychis*
	R. yanglingense	*Amphicarpaea, Cornnilla, Gueldenstaedtia*
Sinorhizobium	*S. abri*	*Abrus precatorius*
	S. americanus	*Acacia spp.*
	S. arboris	*Acacia Senegal, Prosopis chilensis*
	S. fredii	*Glycine max*
	S. indiaense	*Sesbania rostrata*
	S. kostiense	*Acacia Senegal, Prosopis chilensis*
	S. kummerowiae	*Kummerowia stipulacea*
	S. medicae	*Medicago truncatula, Medicago polymorpha, Medicago orbicular*
	S. meliloti	*Medicago, Melilotus, Trigonella*
	S. morelense	*Leucaena leucocephala*
	S. saheli	*Acacia, Sesbania*
	S. terangae	*Acacia, Sesbania*

1.1.7 *Bradyrhziobium japonicum*

The *Bradyrhizobium* genus was described by Jordan in 1982 [48]. It currently consists of 5 species (Table 1-4). *B. japonicum* is a Gram negative, aerobic bacteria, nitrogen-fixing bacterium which develops a symbiosis with the soybean plant (*Glycine max*). Some researches reported that other species such as *Sinorhizobium. fredii*, and *B. elkanii* could act as inocula with *Glycine max* [43,48], *B. japonicum* is mainly used in agricultural lands in Iran, especially in the south west, therefore this study has focused on that species.

The *B. japonicum* belongs to the family *Rhizobia*ceae which includes other nitrogen-fixing bacteria that develop symbiosis with legumes. Strains of *Bradyrhizobium* grown in the laboratory are shaped like short rods, and they generally measure 0.5 to 0.9 μm wide and 1.2 to 3.0 μm long (Figure 1-7).

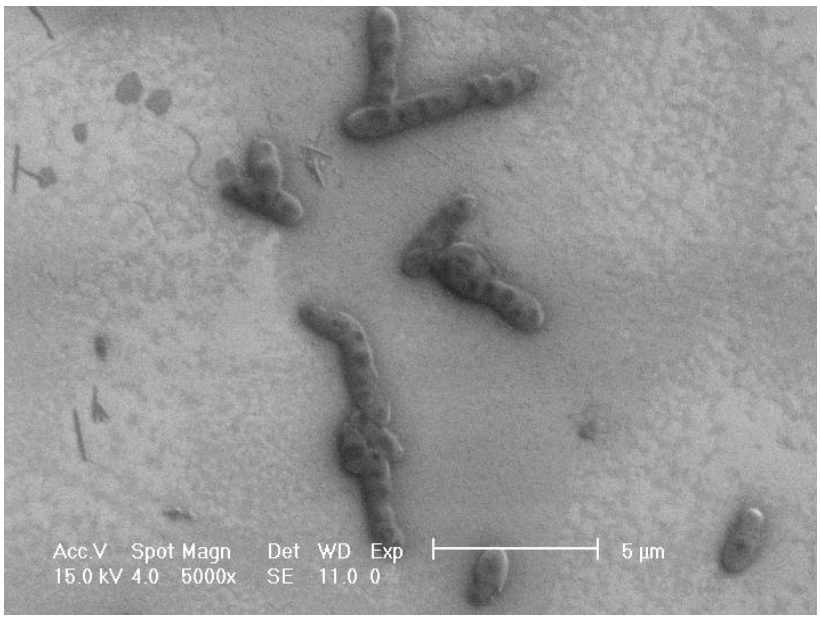


Figure 1-7. Scanning electron micrograph of *Bradyrhizobium japonicum*.

1.2 Symbiotic nitrogen fixation (SNF)

After photosynthesis, nitrogen fixation is the second most important feature sustaining life on Earth. The product of fixed nitrogen is ammonia which furnishes the two main groups of macromolecules like proteins and nucleic acids. The nitrogen molecule has a triple bond and it is not useable by the plant, although the nitrogen is necessary for plant growth. Thus it must be "fixed" (combined) in the form of ammonium (NH_4^+) or nitrate (NO_3^-) ions. This process is called nitrogen fixation mechanism. Nitrogen fixation needs energy for breaking the triple bond, as the nitrogen molecule (N_2) is quite inert. To break it apart so that its atoms can combine with other atoms requires the input of substantial amounts of energy.

History of the discovery of the SNF by organisms express that probably since the time of Egyptians, farmers have been known that the legumes such as pea, lentil, and clover have fertility effect on soils, so that for millennia and the Romans have known, such practices as green manuring, crop rotation, and mix-cropping [103]. Hirsch report that in 1888 Hermann Hellriegel with Hermann Wilfarth recognized that the legume root nodules themselves are responsible conversion of atmospheric nitrogen (N_2) to ammonium [103]. Although in 1679 Malpighi published a drawing and thought that “bumps” on legume roots were insect galls [103].

Fixation of nitrogen is process of nitrogen (N_2) to reactive nitrogen (Nr)[†] like ammonium, either by chemical reaction in industry or by bacteria activity [71]. Mechanisms of nitrogen fixation are atmospheric fixation, biological fixation, and industrial fixation (which is called Haber-Bosch process).

The symbiotic fixation was the first economically feasible method, which synthesis NH_4 from H_2 gas and atmospheric N_2 directly. The symbiotic nitrogen

[†] Galloway *et al* .(2004) reported that the term reactive nitrogen (Nr) includes all biologically active, photochemically reactive, and radiatively active nitrogen compoumds in the atmosphere and biosphere of the Earth. Thus Nr includes inorganic reduced forms of nitrogen (e.g., NH_3, NH_4^+), inorganic oxidized forms (e.g., NO_x, HNO_3, N_2O_3, N_2O, NO_3^-), and organic compounds (e.g., urea, amines, proteins, nucleic acids), please see reference [71].

fixation (SNF) occurs in swelling root hair cells, which is called a nodule. The SNF process can be represented by the following equation, in which two moles of ammonia are produced from one mole of nitrogen gas, at the expense of 16 moles of ATP and a supply of electrons and protons (hydrogen ions):

$$N_2 + 8H^+ + 8e^- + 16\ ATP \rightarrow 2NH_3 + H_2 + 16ADP + 16P_i \qquad (1\text{-}1)$$

This reaction is performed exclusively by prokaryotes (the bacteria and related organisms), using an enzyme complex termed nitrogenase. The ability to fix nitrogen is found only in certain bacteria. Some bacteria live in a symbiotic relationship with plants of the legume family (e.g., soybeans, alfalfa), and other bacteria establish symbiotic relationships with plants other than legumes (e.g., alders), and at least some nitrogen-fixing bacteria live free in the soil [25,48].

1.2.1 Nitrogenase enzyme

The nitrogenase catalyses the conversion of nitrogen gas to ammonia in nodules. In legumes it only occurs within the bacteroids. Three major types of nitrogenase have been identified: one containing molybdenum with iron, the second containing vanadium with iron, and the third apparently containing iron only. Increasingly more accurate structural studies have determined that all nitrogenases consist of two separately purifiable proteins (Figure 1-8). The larger of the two has a molecular mass of 230 kDa and is a molybdenum–iron (MoFe) protein. This protein is believed to be the site of substrate binding and reduction. The smaller protein has a molecular mass of 60 kDa and is an iron (Fe) protein. Its function is the donation of electrons to the MoFe-protein to facilitate dinitrogen reduction catalysis as per the reaction (see equation 1-1, and Figure 1-8) [48,231]

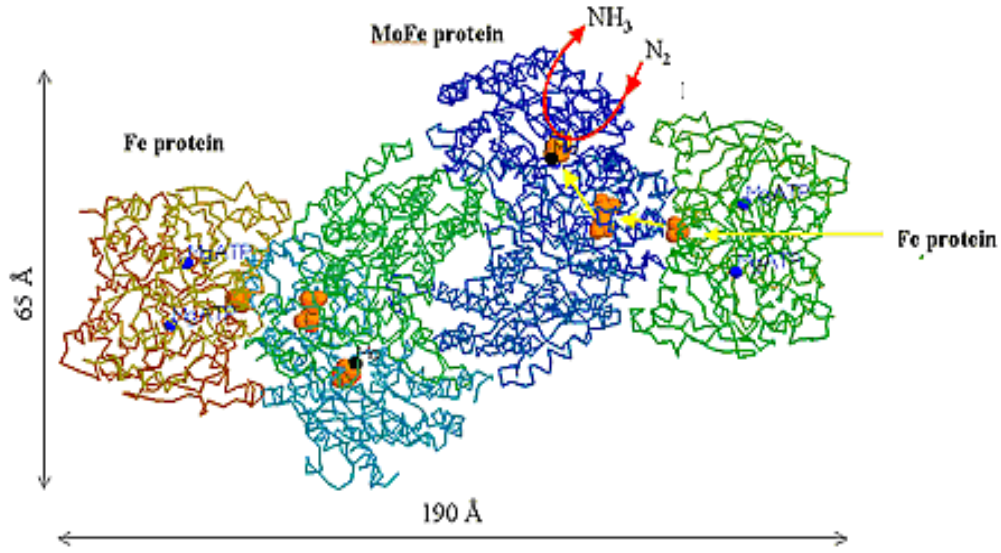


Figure 1-8. Scheme of the nitrogenase structure [231].

1.3 Nodulation

The nodules are the micro–aerobic niche for the nitrogen fixation process, protecting the bacterial nitrogenase from inactivation by oxygen [48]. Nodule organogenesis in the host root starts when micosymbionts intracellularly infect the host cell. Based on ontogeny nodules differ in legume species and they are categorized into determinate and indeterminate nodules. Determinate nodules are indentified in the tropical and subtropical legumes such as soybean, cowpea, common bean and lotus and these nodules are spherical, originate from root outer cortical cells and lack a persistent meristem, whereas temperate legumes produce nodules which are cylindrical, originate from root pericycle, inner cortical cells, and have a persistent terminal meristem; e.g., alfalfa, pea, and white clover [238]. Successful infection is due to mutual excretions of plant roots and *rhizobia* cells. Excretions of host root and *rhizobia* act as signal molecules and they are the most important factors for establishment of nodules. In fact signal molecules determine the host specificity of *rhizobia* for particular legumes include flavonoids secreted by the host plant roots and the lipochitooligosaccharides (LCOs) by *rhizobia*.

1.3.1 Nodule establishment in brief

The nodulation process (Figure 1-9) depends on compatible signal molecules between symbiotic partners.

Exudates of host root and *rhizobia* colonization, (step 1): each legume species produces a distinct mixture of flavonoids and isoflavonoids, which their quantity and the spectrum of the different compounds and vary with age and the physiological status of the plant growth [177,178,235]. For example mostly genistein and daizein isoflavoids are released from soybean root.

Response of compatible *rhizobia*, (step 2): such flavonoids are perceived by the *rhizobia* as positive signals to which they respond by the production and release of signal molecules called nodule factors (nodf), *nod* genes are expressed by specific isoflavonoid signals to make the nodf. The nodf differ in the *rhizobia* species and only compatible nodf can induce nodule establishment in the host root, with very low concentrations (e.g., 10^{-12} M) [177]. For example, the LCOs secreted as the nodule factor from *B. japonicum* recognizes genistein (an isoflavonoid) as the signal from soybean root.

Attachment (steps 2 and 3): “the tip of emerging root hairs is the primary target for infection by *rhizobia*, probably because their thinner and less cross-linked cell walls allow for the re-arrangement of underlying microtubules, changing vesicle trafficking to the growing tip and thus better enabling subsequent penetration by *rhizobia*. Attachment of *rhizobia* to root hairs stimulates root hair deformation within 6-8 h, and also promotes cortical cell divisions” [60].

Infection thread (IT), (step 4): when root hair entraps *rhizobia* by root hair curling, the *rhizobia* can enter via two main ways such as the root hair or through cracks in root epidermal tissue (Figures 1-9 and10) [60,178]. Mostly the *rhizobia* infect via root hair and make infection threads which have microtubular structure and consist of host cell wall components that act as as a passage for microsymbionts into cortical cells of the host [70, 60].

Penetration of the IT (steps 5 and 6): probably because of an enriched nodf concentration and cell wall–degrading enzymes in encapsulated *rhizobia* within cortex cells, the IT penetrate into host cell wall (not in plasma membrane) by resynthesis and redigestion [60]. “This recurring cycle coupled with viscous extracellular matrix embedding of the microcolony and continued bacterial growth process a ‘forward’ pressure that is needed to ‘push’ against the root hair turgor pressure, then these dynamics of this process results in the formation of the plant-cell wall derived infection thread and filled with proliferating bacteria embedded in the ever-hardening extracellular matrix” [70].

Nodule development (steps 7 and 8): the *rhizobia* are released into cortex of host cells (proliferating nodule meristem cells) and they are enclosed in peribacteroid membrane (PBM), and induce dividing cells [60].

Nitrogen fixation, (steps 9 and 10): nodule vascular tissue connect to root vascular bundles and root cells and nodules exchange essential nutrients via xylem and phloem, finally bacteroids start to fix atmospheric N_2, which is converted into ammonia [60,177]. When legume and microsymbionts are well matched, the effective nodules are deep red inside, whereas ineffective nodules are green or white (Figures 1-9 and 10).

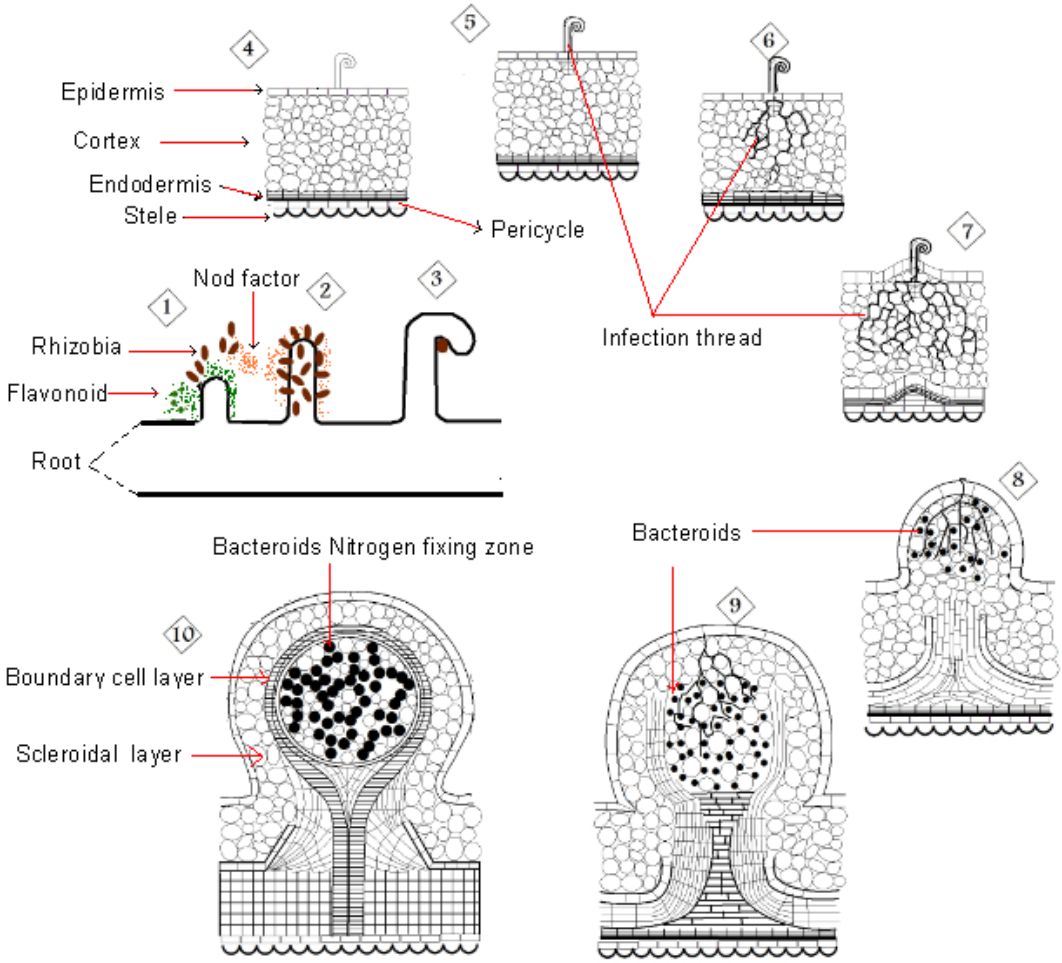


Figure 1-9. Scheme of nodulation process.

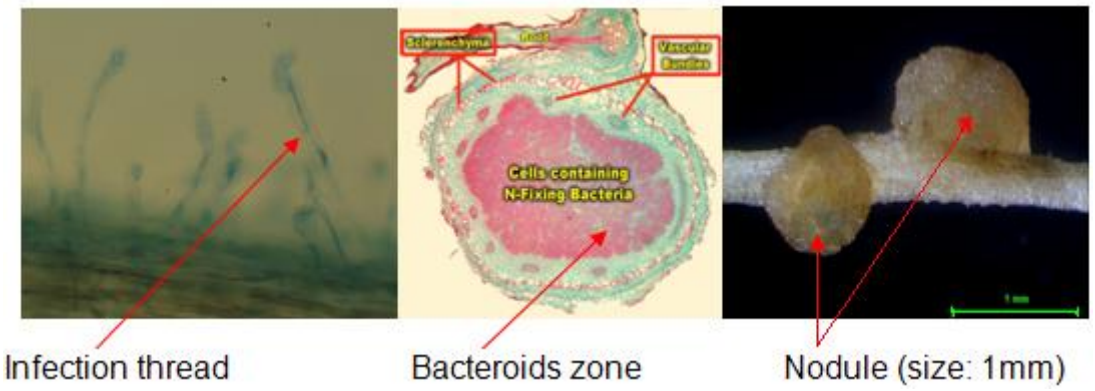


Figure 1-10. Nodule structure, infection thread, and bacteroid zone.

1.3.2 Signal molecules in nodulation

Basically symbiosis between microsymbionts and host root is a molecular dialogue between the symbiotic partners that depends on the specific recognition of signal molecules produced by *rhizobial* bacteria and host root hair cells. Primary signal molecules are lipochitooligosaccharides (LCOs), flavonoids, and secondary signal molecules include auxin, cytokinin, ethylene, and some genes like *Enod40* which is essential for the initiation of cell divisions in the nodulation [19, 48].

1.3.2.1 Lipochitooligosaccharides (LCOs)

The LCOs as nodulation factors (nodf) are produced by *rhizobia* such as *Allorizobium*, *Azorhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Rhizobium*, and *Sinorhizobium* [19, 230]. These nodf are signal molecules responding to secreted flavonoids from host root. The LCOs play a key role during initiation of nodule development and penetration of infection thread (IT) into host cell wall [19, 95]. These signal molecules trigger the cortical cell division that result in nodule formation [131]. The nodf act at concentrations as low as 10^{-9} to 10^{-12} M, and particular substituents (e.g., acetyl group, methyl, and carbamoyl) protect against nodf hydrolysis by enzymes of host plant origin [95]. Mostly the LCOs and *rhizobial* cell surface polysacchardies such as lipopolysacharides (LPS) and lipopolysacharides (EPS) are involved in attachment, penetration, and invasion of emerging nodules by microsymbionts [19, 95,131].

The nodf are composed of an oligomeric backbone of three to five β-1,4-linked *N*-acetyl-D-glucosamines that are *N*-acylated on the nonreducing end residue and carry various substitutions on the reducing and nonreducing teminal glucosamine (GlcNAc) residues (Figure 1-11) [230].

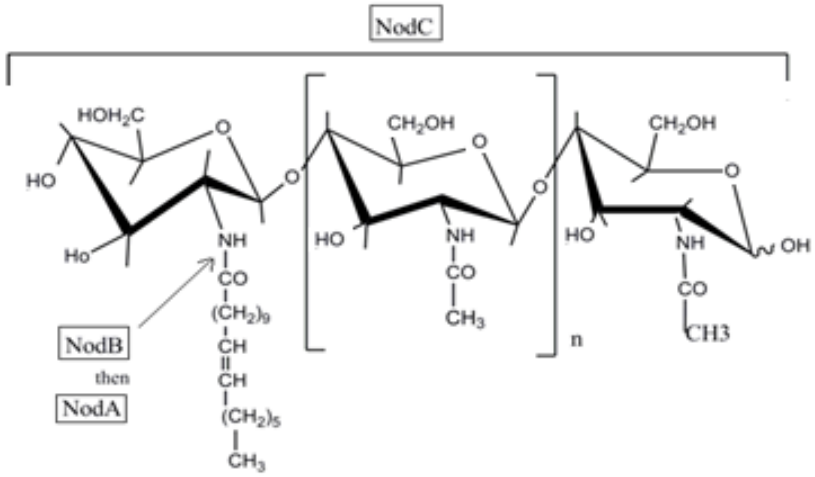


Figure 1-11. Generalized structure of a nodf: NodC is a *β*-glucosaminyl transferase, links the UDP-*N*-acetyl glucosamine monomers into a chitin-like backbone. NodB removes an acetyl group from the terminal residue of the chitin oligomer. Then, NodA catalyzes the transfer of a fatty acyl chain onto the resulting free amino group, using acyl-ACP from fatty acid biosynthesis [104].

Each species of *rhizobia* has a set of nodulation genes of which five (*nodABCIJ*) are common for all [48]. The basic structure (backbone) of LCOs is synthesised by *nodABC*, while *nodIJ* are involved in their secretion [48]. NodC produces chitooligosaccharides, NodB remove the N-acetyl group from nonreducing terminal residue, and NodA is involved in the transfer of the acyl chain [150]. Furthermore many of the other Nod proteins are involved in the attachment of various substituents, and also modification of the LCO core depend on *nod* genes that are strain-specific [209].

1.3.2.2 Isoflavonoids

Flavonoids are polyphenolic secondary metabolites that are secreted from plant roots [104, 266]. Based on their chemical structure they are categorized into flavonols, flavones, flavanones, isoflavones, catechins, anthocynaidins, and chalcones [19, 266]. The core of the flavonoids consists of two benzene rings linked through a hetrocyclic pyran or pyrone ring (Figure 1-12) [104].

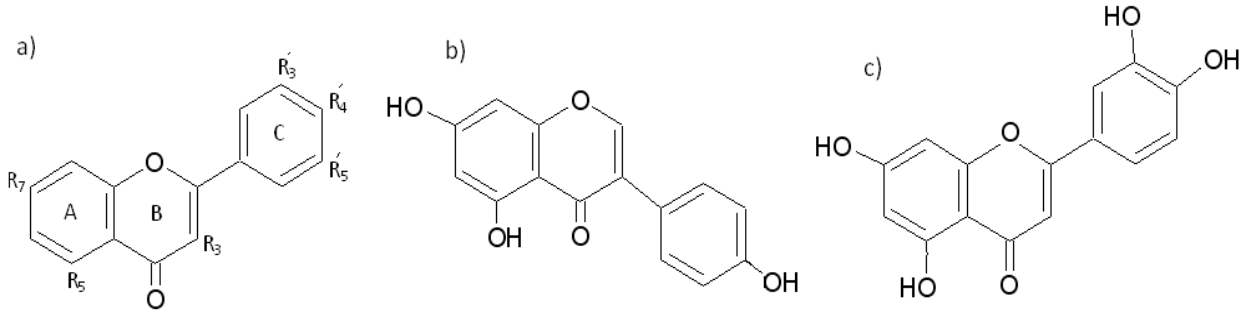


Figure 1-12. Chemical structure of flavonoids: (a) is the generalized structure of a flavonoid, which changes in the R groups lead to different kinds of flavonoids; (b) is an isoflavonoid (genistein); (c) is a flavone (luteolin) [104].

In fact several specific functional groups (e.g., hydroxyl and dihyro) on the ring produce different flavonoids like isoflavone which differ from flavones in location of phenyl group [19, 104].

Legume plants are the main source of isoflavonoids, which have a dual role in the symbiosis between *rhizobia* and host root, so that first they can act as chemoattractants for the microsymbionts and inducers of *nod* gene expression, and second during disease response they are induced to provide defence compounds [104]. For example, the isoflavone daidzein is a precursor of phytoalexins including medicarpin and glyceollins, and also the isoflavone genistein has antifungal activity and is a precursor of phytoalexin kievitone [266]. Mostly isoflavonoids such as genistein and daidzein are produced by soybean and induce effectively *B. japonicum nod* genes, whereas they inhibit *S. meliloti nod* gene expression [104]. Interestingly this specificity enables *rhizobia* to recognize their host plant roots from those of other legumes. Therefore, being a specific flavonoid not only induces nod gene expression, but also *rhizobia* chemotaxis [104].

Genistein is one of the major isoflavonoid compounds in soybean seed and soybean root exudates. It is responsible for inducing the expression of the *B. japonicum nod YABC* operon [108,133, 202]. Thus in turn synthesised LCOs trigger genistein secretion by soybean root, in fact this feed back induce to synthesis of the genistein pathway [19].

1.3.2.3 Secondary signal molecules

1.3.2.3.1 Auxin

Auxins as mitogenesis signals are phytohormones; their role is morphogenesis and control organogensis in plant like nodule development [48]. In fact after the LCOs perception during *rhizobial* infection auxin is synthesised and distributed in the cortex opposite the site of infection to induce mitosis and control cell cycle and cell proliferation in plant root hair for nodule primordial development.

There are two major nodule types (determinate and indeterminate), and the accumulation and distribution of the auxin differ in the determinate and indeterminate nodules. For instance, soybean roots have determinate nodules, and auxin maximum only is in the middle cortex, whereas in the indeterminate nodules (e.g., *Medicago* sp.) first accumulated and distributed in all cortical and vascular cell [160]. However, the nodf and likely flavonoids inhibit auxin flow and inhibit induction of root acropetal auxin-transport [48,49].

1.3.2.3.2 Cytokinins

Cytokinins are essential compounds for plant growth. Their structure resembles adenine and they have similar functions to kinetin [13, 72]. The main source of cytokinin is the root tip [178]. After nodf perception at the epidermis the distribution and accumulation of the cytokinins in the cortex increase, which leads to promote nodule organogensis in the cortex and stimulate cell division [49]. The effect of cytokinins is to initiate nodule development in cortex while suppressing lateral root formation in the pericycle cells [49]. The numbers of nodules formed reduce due to decreasing cytokinin level [149].

However, analysis of *B. japonicum* tRNA showed at least three cytokine nucleosides [91]. Cytokinins in the culture filtrate might be released from the tRNA during cell isolation or by cell autolysis in the culture [91]. In addition microorganisms which form symbiotic or parasitic associations with host plants produce free cytokinins (not located in tRNA molecules) [91]. In fact previous researches suggested that *nod AB* proteins are involved in the production of a

factor that stimulates cytokinesis in plant proplasts, although the mechanism still is unclear [85, 115, 220, 224].

1.3.2.3.3 Ethylene

The ethylene signal mediated by the gaseous phytohormone ethylene involves nodulation and regulates many aspects of the symbiosis interaction between host and bacteria (e.g., regulate formation of the infection thread, inhibit the maintenance of calcium spiking after induction by nodf, regulate the placement of the nodule primordium in the root by inhibiting cell division, and regulate nodule number; although these mechanisms are still unclear) [179]. In fact inoculation with *rhizobia* microsymbionts or the nodf induces ethylene production by root [96].

Effect of ethylene signal depends on nodule type (determinate or indeterminate) and infection mode [48]. Ethylene effects can be classed as negative regulator, having no influence, or being essential. For example, nodulation of soybean and bean is indifferent to ethylene, or ethylene has a negative regulator in indeterminate nodules, and/or is required for *rhizobial* infection and nodulation of aquatic legume *Sesbania rostrata* [48].

1.3.2.3.4 *Enod40*

In nodulation many genes are induced in particular cell types or nodule tissues [83, 233]. So far among them only *Enod40* emerges with a regulatory role in nodule initiation [83, 233, 48]. In fact the *Enod40* encode two small peptides (12- to 13-amino acid peptide and a 3´ RNA). Its secondary structure is a key element in the signalling processes underlying nodule organogenesis [83, 233, 48]. The *Enod40* is expressed in the early stages of the infection in the legume roots (before the initiation of cortical cell divisions that lead to nodule formation) and mainly in pericycle close to the protoxylem pole [83, 233]. In addition *Enod40* is induced by nodf, chitin pentamer and phytohormone cytokinin [83, 233]. Furthermore, expression of the *Enod40* is induced in meristems and the developing vasculature of lateral organs [48].

1.4 Agrinanotechnology

The successful achievements of nanotechnology in medicine under in vitro conditions have generated some interesting ideas in agrinanotechnology. Progressing in nanodevices and nanomaterials provide novel techniques in the development of plant biotechnology, microbiology and agriculture. To date researches in nanotechnology were mostly in abiotic field like electronics and energy, and also in medicine and life science, although their achievements and experiences facilitate promotion quality and quantities of agricultural products.

Briefly, answering the question: “what is nanotechnology?”, Ramsden (2005) proposed that nanotechnology is the science of design and fabrication of materials, devices and systems with control at nanometer (10^{-9} m) dimension [200]. The main serious current problem in development and agriculture policies is that productivity increases tend to be quantitative while decreasing quality values could annihilate enhancing quantitative values and reducing costs [201]. The application of nanotechnology in agriculture could proffer various ways of solving problems of famers. Agrinanotechnology widely disseminate advances in agricultural researches, such as reproductive science and technology, conversion of agricultural and food wastes to energy and other useful by–products through enzymatic nanobioprocessing, disease prevention and treatment in plant using various nanocides [218].

However, the synthesis of nano-iron, iron being one of the most important and effective elements in the symbiotic nitrogen fixation process, was one of the objectives of this study for the enhancement of symbiotic nitrogen fixation efficiency under environmental stress. The main advantages of using magnetite (Fe_3O_4) nanoparticles are that they can provide the metal required to make essential metalloproteinases (e.g., leghemoglobin and nitrogenase) in root nodules, scavenge radical ions and radical molecules secreted from bacterial cells in the medium under extreme conditions to prevent of damaging *rhizobial* cells, prevent shifting pH (liquid culture medium pH is changed by consumption of constituents and/or molecules secreted), indirectly regulate

oxygen in the bacteroids by leghemoglobin proteins (Fe is involved in the composition of nodule key protein like leghemoglobin, thus under iron deficiency and/or salinity stress the nanoparticles could compensate iron deficiency for constructing the leghemoglobin) [48, 153]. In addition magnetite nanoparticles permit a very specific localization of the particles to release their load, and these nanoparticles as iron fertilizer could be use in high minimum concentration, and they could compensate iron deficiency for plants [151,173].

1.5 Hypotheses and objectives

Bradyrhizobium japonicum viability under extreme conditions is a key factor for formation of nodules on the roots, normally resulting in the maximum symbiotic nitrogen fixation (SNF) [18,48,74,89,88,203]. The viability decreases under extreme pH, salinity in liquid media and desiccation time on the seeds, due to declining of available oxygen, water activity (a_W) and oxidation reaction potential(ORP) [7,9,22,90,107,217,235,236,243]. During bacterial growth in a medium some materials are secreted, which can affect the number of the viable cells (e.g., oxidizing agents like O_2^-, H_2O_2, and $HO^{\cdot}$), then the optimum pH for bacteria growth changes toward greater acidity or alkalinity, and the direction of variation depends on the genus and strains [81,203,204]. Normally *Bradyrhizobium japonicum* changes the pH toward alkalinity [7,9]. Therefore if *Bradyrhizobium* used in alkaline soil, then decreasing the SNF will be expected. Thus the approaches, which can effect on the secreted materials of bacteria and control media conditions, are important for enhancing of the SNF under extreme condition.

However, recently magnetite particles have been widely utilized for promotion and optimization of biological mechanisms [76,77,78,79,173,176,201, 270]. When the particles are in nanoscale they are able to act like enzymes, buffers and catalysts, for instance, reinforcing defence mechanisms of microorganisms in environmental stress, and the remediation natural resources [181,274].

On the other hand there is not enough evidence for the benefits of application of magnetite nanoparticles as an optimizating agent for *Bradyrhizobium japonicum* growth under extreme conditions. Hence experiments were conducted in the lab to answer the following research main questions:

1. What type of fabricated magnetite nanoparticles have a positive effect on the *Bradyrhizobium japonicum* growth?

2. What quantity of the nanoparticles can preserve optimum medium conditions?
3. How many viable bacterial cells survive in liquid media and on the seed when the *Bradyrhizobium* is coated by nanoparticles?
4. What amount of nodule factors like lipochitooligosaccharides (LCOs) is induced to be secreted from *Bradyrhizobium japonicum* by nanoparticles?
5. What amount of flavonoids like genistein is induced to be secreted from soybean roots by nanoparticles?
6. How much nitrogen is fixed in nodules of *Bradyrhizobium japonicum* coated with nanoparticles?

This study was conducted to test the following hypotheses based on the above mentioned questions:

1. Magnetite nanoparticles considerably increase *Bradyrhizobium* growth rate (BGR)
2. Magnetite nanoparticles decrease deletrious effects on the BGR under extreme conditions
3. Coating *Bradyrhizobium japonicum* with nanoparticles can contribute fixed nitrogen to the ecosystem's nitrogen pool.

Based on the hypotheses above, the following main objectives were formulated for this research work to determine the:

1. Effect of fabricated magnetite nanoparticles (which are unmodified or modified with some polymers) on the BGR.
2. Desirable quantity of the nanoparticles to maximize bacterial growth under extreme conditions (pH and salinity).
3. *Bradyrhizobium* cells viability on the seed during desiccation and ar different temperatures.
4. The percentage of nitrogen derived from the SNF of soybean plant.
5. Total dry biomass of the soybean plant.

6. The potential SNF-nitrogen contribution of soybean plant to the ecosystem’s nitrogen pool.
7. To investigate the mechanisms by which the magnetite nanoparticles influence SNF-nitrogen.

2 Literature review

2.1 Effect of environmental stresses on soybean plant, *rhizobia*, and symbiotic nitrogen fixation (SNF)

The term stress is derived from the Latin word "*stringere*", which means a constraining or impelling force, and stress is defined as "the external constraints which restrict the rate of dry matter production of all or part of the vegetation below its genetic potential" [125].

From planting a seed in soil to harvest a plant, growth and development of plant is subjected to environmental stresses. Thus crop productivity depends on effect of environmental stresses. Soybean yield potential is defined as the maximum yield of a crop cultivar grown in an environment to which it is adapted with nutrient and without environmental stresses (e.g., temperature, pH, salinity, and water), and pests and diseases effectively controlled [61, 186].

Dilworth *et al.* (2008), categorized environmental stresses according to three types: i) Biological stress (host response against an infection microorganism); ii) Chemical stress such as pesticides, herbicides, fungicides, heavy metals, hyper- or hypo-osmosis, pH (acid or alkaline or pH shift), reactive oxygen species (ROS), reactive nitrogen species (RNS), nutrient limitation; iii) Physical stress such as high or low temperature, water stress (desiccation), ultraviolet light [48]. Response stress in cells may be triggered by a common or specific signal and may occur at the transcriptional, translation, or proteolytic level [48]. Furthermore a given stress may also have more than one effect: e.g., salinity may act as water stress, later that can affect photosynthetic rate and deprive the host from photosynthates, consequently will decrease transferring assimilates toward nodule and bacteroids, which is necessary for bacterial growth and proliferation, or may affect nodule metabolism directly. By 1999, H. H. Zahran said "the most problematic environments for *rhizobia* are marginal lands with low rainfall, extremes of temperature, acidic soils of low nutrient status, and poor water-holding capacity" [268].

2.1.1 Temperature stress (high or low temperature)

High or low temperature in soil or medium inhibits *rhizobia* growth and host plant growth, consequently decreases symbiotic nitrogen fixation through its effect on nodulation, metabolism reaction in nodules and nitrogenase activity [156,165,172,273].

2.1.1.1 Response of soybean plant to temperature

Development and productivity of soybean depends on temperature Soybean is a warm season crop and its temperature requirement differ in the growth and development phases. Investigations show that under adequate supply of soil moisture and oxygen, the water uptake and the rate and percentage of germination of viable seeds is dependent on soil temperature [255]. 10-15 °C has been suggested for the minimum temperature requirement of the germination [130,264], and optimum temperature germination is approximately 24 to 28 °C. In addition 28 to 30 (day) and 15-18 (night) are optimum during growth and development of soybean.

Some researches argue that high temperature reduce quality of soybean seed [124]. Soybean seed germination decreased linearly with increased day/night temperature [124]. In tropical zones, high temperature and relative humidity deficit during the final stages of seed development and maturation are serious seed production problems that result in rapid loss of viability in the standing crop [124]. Reproductive growth in grain legume species is the most important phase of the plants growth, and consequently it leads to grains formation. In contrast with vegetative growth and development, temperature requirement of the reproductive phase is lower than vegetative growth, for example, flowering initiation of soybean was reduced by temperature higher than 32 °C, and seed formation was delayed at 30-40 °C [250].

Furthermore soybean plants were exposed to temperature of 35 °C for 10 h during the day, the yielding reduced about 27% [250]. Increasing night temperature more than 18-20 °C brought the flowering time forward and

decreased seed size and seed yield [276]. However, more evidences show that the reduction of seed yield would be by a high day temperature than night temperature [82,276].

Physiologically, the high temperature stress during reproductive development many have affected flower abortion, sequent sink site, and later pod abscission result a decreased number of seed per plant [171,250]. In addition high temperature have negative effect on cell expansion, cotyledon cell number and thus seed filling rate, resulting in the lowered weight per seed [171,250].

Soil temperature between 30 and 33 °C caused little change in the nitrogen fixation, but temperature above 34 had a negative effect [123]. By soil temperature >35 °C, root development and nodulation of soybean was limited [123, 165].

Low soil temperature is potentially an important factor limiting soybean growth and yield [273]. By low temperature (10 °C) photosynthesis reduced [247], this reduction of photosynthesis due to changing in the properties of mesophyll chloroplasts [247]. Low soil temperature effect causes to delay the steps of nodulation [167]. Likewise the expression of the *nod* genes will be suboptimal at low temperature, resulting in delays in the onset of nodulation [273].

Moreover, temperature stress cause to decrease photosynthesis activity, and hence productivity of the plant decrease, then translocation of photosynthates to root and nodules will diminish, resulting in decrease in the mechanism of symbiotic nitrogen fixation.

2.1.1.2 Respose of *rhizobia* to temperature stress

Adaptation and response of *rhizobia* to stress conditions is one of the important physiological processes of bacteria that determine viability and bacterial growth rate. Different strains of *rhizobia* have different capacities to

withstand temperature stress. Previous studies showed that genetic evidence suggests that environmental stress even occurs when *rhizobium* cells are released into root hair cells following infection [48,268].

Marsh *et al*. (2006) reported that the growth of *rhizobia* in media under extreme temperature influence their symbiotic interaction with host legumes [156,165,215,268,272]. Some researches show that death of *rhizobia* may occur above 37 °C [111]. Temperature stresses probably cause to change physiological and genetic modification in bacteria (e.g., plasmid deletion) and genomic rearrangement [111].In general low temperature (below 10 °C) cause to limit growth of *rhizobia* [53]. However some of *rhizobia* species, which have been isolated from subarctic and arctic regions showed better growth, earlier nodulation and higher nitrogenase activity with clover at 10 °C [195].

High soil temperature (above 35 °C) limits some *rhizobia* species like *Rhizobium leguminosarum* bv. *phaseoli*, and it unable to fix nitrogen in symbiosis with common bean [109]. However other species of the *rhizobia* and *Bradyrhizobia* (e.g., *Leucaena leucocephala*, and *Prosopia juliflora*) can resist high temperature above 35 °C [109].

Moreover previous studies show that *rhizobia* could diminish or control effects of temperature stress [144,165,174,183,210]. In addition tolerance to temperature stress depends on how *rhizobia* experience extreme temperature. For example, Michiels *et al.* 1994 suggested that the themotolerance under 50 ˚C temperature stress observed after a preincubation at 45 ˚C which is related to the synthesis of heat shock proteins [165]. Munchbach *et al.* (1999) reported that there are heat shock proteins in *B. japonicum* due to extreme temperatures [170]. Nadal *et al.* (2005) suggested that high temperature changes metabolic activity in *rhizobia* (microsymbionts), accordingly heat shock proteins (hsps) are produced in the cellular envelope polysaccharides and cellular proteins [174]. Although the basis of thermotolerance in *rhizobia* strains is unclear, previous studies suggested that the themotolerance is related to bacterial cell surface properties (exopolysaccharides and lipopolysaccharides) and protein profile

[144,174,183,210]. The heat shock proteins mainly prevent protein aggregation, assist refolding, bind to denatured proteins and target misfolded proteins for degradation under extreme temperature stress and conserve them in a folding-competent state [170,174].

2.1.1.3 Respose of symbiotic nitrogen fixation (SNF) to temperature stress

Maximum soil temperatures in the tropic regularly exceed 40 °C at 5 cm and 50 °C at 1 cm depth, and can limit nodulation [111]. High temperature indirectly effects on the metabolism of the host plant, and directly effects on the SNF [111]. Optimum temperature for the SNF of soybean in the root zone is 25-30˚C [148,165,172,273]. Both high and low soil temperatures delay nodule formation on the roots [165]. Significant fluctuations of temperature are a major problem for nitrogen fixation for *Bradyrhizobium* strains. Thermal stresses caused decreased expression of the nodule genes, and also decrease development of root hairs and decrease attachment of *Bradyrhizobium* on roots [54,271]. For example, low temperature (suboptimal) stress caused decreased *Bradyrhizobium* motility toward root hair cells and taking resulted in curling root hair and delay in nodule formation [271].

Researches argue that the root infection process is may the component most affected by high temperature, with sensitivity located at the nodulation sites [111] High temperature inhibits root hair formation, and reduces the number of sites for nodulation, attachment of bacteria to root hairs, root hair penetration, and infection thread formation [111]. Likewise high temperature effects on the nodule initiation and bacteroid development [111]. The exchange of molecular signals between host plants and *rhizobia* is also affect by high temperature. Some investigations show that at 39 °C, the release of nodule genes inducers from soybean and common bean roots was decreased [111,261] Also Zhang and Smith (1996) showed that secreted genistein (as a signal molecule) of soybean root varied due to low soil temperature [271]. Zahran (1999), and Michiels *et al.* (1994), showed that SNF in soybean decreased at 35 and 40 °C, and also its nodulating stopped at 42 and 45 °C during 12-h and 9-h days,

respectively [165,268]. But the critical temperature is at 30 °C for N_2 fixation of clover and pea [165]. Roughley *et al.* (1981) showed that temperature stress affects root hair infection, bacteroid differentiation, nodule structure, and the functioning of the legume root nodule [216].

2.1.1.4 Thermal stress: conclusions

i) Decreasing yield of soybean seed

ii) Photosynthate deprivation.

iii) Decreasing expression of the nodule genes.

iv) Decreasing root development.

v) Decreasing attachment of bacterial cells on the root.

vi) Takes a longer time for root hair curling, infection and nodule formation, bacteroid differentiation.

vii) Disordering in the exchanging of signal molecules between host and *Bradyrhizobium* strains.

viii) *Rhizobia* at the threshold of extreme temperature stress via changing metabolic activities in the cell produce heat shock proteins (hsps) to decline or control the impacts of stress.

ix) The hsps via prevention of protein degradation, assisting refolding and binding to denatured proteins preserve structural molecules of *rhizobia* under temperature stress.

2.1.2 pH stress (acid and alkaline)

Alkaline and acidic soils are a serious challenge facing agricultural production in many arable lands of the world, which remarkably decrease legumes productivity [214,243]. Previous results showed that the maximum yield and growth of legume plants and fixed nitrogen by *rhizobia* was obtained at pH=7 and 6.5 [7,9,48].

2.1.2.1 Response of soybean plant to pH

Soil pH effects on nitrogen ion availability, uptake and assimilation by plants [98]. Both acidic and alkaline soil pH constraint growth, development, and production of soybean [38, 98,189,205,241,242,244]

Soil acidity is one of the important constraint factors that limit crop growth [31,98,189,205]. At low soil pH (2.5 and 3.5) soybean plants showed leaf scorching and yellowing, and also produced pods, which were without seed [205]. Soybean is more sensitive to acidic soil than other legume plants like peanut [189]. Acid soils cause decreased shoot and root dry weights, nodules dry weight and number of nodules, shoot nitrogen concentration, and chlorophyll levels of inoculated soybean plants (without using nitrogen fertilizer) [38,31,98]. Some properties of acid soils (pH<4) are highly soluble or exchangeable manganese and aluminium such that they are able to produce toxicity in many crops, and also reduce their productivity [189]. In addition acidic soil (pH<5) cause to decrease some nutrients availability in crop filed, (e.g., calcium and magnesium deficiency and poor phosphate availability) [5,189]. Some evidences show that acidic soils via hydrogen ion toxicity limit nodulation of soybean [38]. However, acid soil toxicity could not be a single factor, but a complex of factors (e.g., nutrient stresses) that reduce crop yield.

Previous studies [241,242,244] suggested that most legume species decreased shoot growth, nodulation and nitrogen concentrations in shoots under alkaline stress (pH≥8). But alkaline stress tolerance depends on plant species and *rhizobia* species, so that nodule numbers of *Lathyrus ochrus*

persist under alkaline stress [242]. Tang *et al.* (2006) found a relation between alkaline stress, iron deficiency and poor nodulation in some legume genotypes [244]. They suggested increasing pH above 7.5 exacerbated the impact of iron deficiency on plant biomass and nodulation, and the response was poor host plant growth (photosynthate deprivation) and poor nodulation [241,244]. In fact alkaline stress declines both iron availability and iron uptake by host roots under high pH soil [121,126,240]. In addition iron is directly involved in nodule formation and the synthesis of the nitrogenase enzyme and therefore any kind of iron deficiency affects nitrogen fixation [244].

There is debate whether the impaired nodulation associated with high pH results from the effect of alkaline stress or iron deficiency induced by alkaline stress or a combination of the two [48]. Tang and Thomson (1996) showed that the nodulation of *Lupinus angustifolius* species was sensitive to pH≥6 [242], while *Lupinus culinaris* was very sensitive to low pH (pH<7) [242]. Therefore research about the impact of alkaline stress on the SNF is important for the development of legumes.

2.1.2.2 Respose of *rhizobia* to pH stress

Such acidic soils conditions pose problems for the bacteria [111]. Optimum pH of the *rhizobial* growth is between 6.0 and 7.0, and relatively *rhizobia* species grow well at less than 5.0 [111]. Previous studies showed that pH<4 caused decreased ability of *rhizobia* to survive [48,21]. In fact acid-sensitive strains display typical pleiotropic defects and also show additional sensitivity to one or more other stresses, such as Zn, Cd, Cu, or azide, for instance the low pH stress induces transporter proteins in the membrane which pumps and increases the concentration of heavy metal elements into bacterial cell which lead to toxicity of metals [48]. Fergusen *et al.* (2002) showed that low pH stress significantly reduced the lipo-polysaccharids (LPS) in the bacterial cell membrane [60]. Graham *et al*. (1994) showed that response to low pH stress in *rhizobia* varies between strains: *Mesorhizobium loti*, *Rhizobium tropici*, and *Bradyrhizobium spp* are more resistant to acidity than strains from the genus

Sinorhizobium [89]. Revee *et al.* (2006) showed that some strains of *Sinorhizobium* are able to resist and adapt in low pH conditions by producing acid tolerance response (ATR), which are specific proteins and they could control acid activation and ion cycling, and decrease degradation of proteins in bacterial cell membrane under low pH accompanied by adding 50 mM of calcium to the medium [206]. Moreover previous results suggested that the fast-growing strains of *rhizobia* have generally been less tolerant to acid pH than strains of *Bradyrhizobium* that are slowly growing [89], although the different response between strains of *Rhizobium* and *Bradyrhizobium* under acidic stress are still not quite clear [89].

Highly alkaline soils (pH>8) tend to be high in sodium chloride, bicarbonate, and borate, and are often associated with high salinity which reduce growth of *rhizobia* species [21]. Researches have shown that some of the *rhizobia* strains (e.g., *B. japonicum*) have limited growth at pH= 8.5 in liquid media and in alkaline soils (pH=8.3) [21, 253,132] However, Brockwell and Holliday (1991) showed that some of *rhizobia* strains (e.g., *R. meliloti*) have been grown well at pH above 7.0 [24].

2.1.2.3 Respose of symbiotic nitrogen fixation (SNF) to pH stress

The efficiency of the SNF process depends on balancing pH within the period of attachment of the bacterial cells on the root, formation of nodules and nitrogen fixing by the bacterial cells in the bacteroids zone. The microsymbionts as partner in the SNF usually are more pH-sensitive than the host plants [111]. Bordeleau and Prevost (1994), and Dilworth *et al.* (2008) showed that symbiosis reaction activity and availability of different nutrients for both host plant and microsymbionts decreased when the pH was less than 4 and greater than 8 [21,48]. Acidic soils effect on the early steps in the infection process, including the exchange of molecular signals between symbiotic partners and attachment to the root [111]. Hungria and Stacey (1997) reported that released of nodule genes inducers by soybean and common bean roots was less at pH 4.5 than at pH 5.8 [110].

Zahran (1999) showed that the pH-sensitive stages are the infection process and the attachment to root hairs [268]. Taylor *et al.* (1991) suggested that colonization of soils and soybean roots by *B. japonicum* may be unfavorably affected at pH< 4.6, which will result in reduced cell numbers and nodulation [122]. Comparing 12 strains of *Bradyrhizobium* for their symbiotic performance with groundnut in acidic soils by Diman Van Rossum *et al.* (1994) showed that some strains were totally ineffective under low pH stress (pH 5.0 to 6.5), while others performed well under acidic stress [214].

2.1.2.4 Response to pH stress: conclusion

i) Acidic and alkaline soils limit productivity and growth of soybean.

ii) High pH stress probably induces deficient iron uptake in the plant root and decreasing availability of iron for microsymbionts.

iii) Acidic stress decreases the ability of *rhizobia* to survive and withstand such an undesirable environment.

iv) Acid-sensitivity appears additional sensitivity to one or more other stresses (e.g., Zn, Cu, Cd and azide).

v) Specific regulator proteins control sensitization to acidity, ion cycling, and protein degradation in cell membrane.

vi) The early stages of nodulation are more sensitive to acidic stress.

vii) Fast-growing strains of *rhizobia* are more sensitive to acidic stress.

viii) Alkaline stress causes poor *rhizobia* growth, and decreased nitrogen concentration in shoots, and decreased nodulation.

ix) There exists a relation (correlation) between the effect of alkaline stress and iron deficiency in the symbiotic nitrogen fixation.

2.1.3 Salinity stress

Salinization means the high accumulation of water-soluble inorganic ions such as: Na^+, K^+, Ca^{2+}, Mg^{2+}, and Cl^-, SO_4^{2-}, and HCO_3^- in the soil [267]. Soils which have an electrical conductivity (EC) of more 4 dS m^{-1}(equal to 4000 μS cm^{-1}) in the soil within 25 cm of the surface, an exchangeable sodium percentage (ESP)< 15, and pH< 8.5 are called saline soil [125]. Dilworth *et al.* (2008) suggested that one-third of the world's arable lands are of high salinity [48]. Approximately all climatic zone types in the world are susceptible to salinization [208]. The salinity stress is of high significance in arid and semi-arid lands and at present salinization seriously invades more than 100 countries [208]. Plant growth, *rhizobia* growth and viability reduce significantly due to salinity stress. In fact, salinity induces osmotic stress within plant cells and nodulating bacteria cells [229].

2.1.3.1 Response of soybean plant to salinity stress

Yields of both grain legumes and forage are limited by soil salinity. Previous results led to the failure of soybean to form nodules under salinity stress being attributed to decreased *rhizobial* colonization and shrinkage of root-hairs [48,254,268]. The most responses of legumes plants to salinity are such as inhabitation of expansion and curling of root hair, and reducing number of nodules [223]. Limitation of nodulating under salinity stress is more related to the physiology of the root hair such as its growth diameter, structure, and curling [167]. Increasing concentration of 1.2% NaCl or more is able to eliminate nodulation process, and also increasing salinity cause to decrease soybean fresh weight and plant height [254]. Researches have shown that nodule activity was less affected by salt than growth and nodulation [226,223].

However, Comba et al. (1998) argues that antioxidant defence system (e.g., antioxidant enzymes such as: ascorbate peroxidise, catalase, glutathione reductase, and superoxide dismutase) of soybean nodules probably

responeded to salinity stress [41], and the antioxidant enzymes will increase due to mild saline stress (50 to 200 mM NaCl). In addition Amirjani (2010) reported that proline contant and diamine oxidase activity were increased in soybean by 50 to 200 mM [4].

Soybean plants in order to enhance their tolerance against salinity stress, they change their morphological characteristics, physiological and biochemical processes [4]. For instance producing and accumulating of free amino acids, especially proline by plant tissue during salt and water stress, and these amino acids act as compatible solutes, and adjust the osmotic potential in the cytoplasm [8]. However, legume species differ in the responses of their tolerances to salinity stress [223].

2.1.3.2 Response of *rhizobia* to salinity stress

Among the *Bradyrhizobium* strains, *B. japonicum* is salt-sensitive [229]. Previous results [48,229,258] showed that osmotic stress and direct ion toxicity especially the in root zone result in decreasing survival and nodulation. Lioret *et al*. (1998) and Ruberg *et al*. (2003) showed that salt stress induces *rhizobia* to change cell morphology [219,145], dimension and modification of their polysaccharides, which are very important in the symbiotic mechanism (like exopolysaccharides (EPS) and lipopolysaccharides (LPS)) [219,145]. Vriezen *et al.* (2007) showed that response to salinity stress vary highly between *Rhizobium* strains [258]. They proposed a general response model of *rhizobia* to salinization based on declining general metabolism after an osmotic upshift.

Likewise, Domingguez-Ferreas *et al. (*2006) reported that osmotic stress lead to the induction of a large number of genes having unclear functions and the inhibition of many genes coding for proteins with known functions [50]. For example, one-fourth of all genes specifically downregulated by salt (NaCl) stress encoded ribosomal proteins. Furthermore other previous studies illustrated that under salinity stress and growth-limiting conditions stressed cells of *rhizobia* accumulate compatible solutes and prefer uptake over synthesis within the cytoplasm (e.g., K^+ ions, glycogen, sucrose, trehalose, maltose,

cellobiose, turanose, gentiobiose, palatinose and amino acids like glutamate and proline). These compatible solutes are osmoprotective. In fact, accumulation of osmoprotectants within the cell provides it with sufficient turgor pressure to prevent cell lysis [48,258].

Breedveld and Miller (1994) reported that in hypo-osmotic conditions, cyclic β-glucans play a major role in osmoprotection of *rhizobia*, so that the production of cyclic β-glucans accumulate in the periplasmic space and protects cells under hypo-osmotic environments via declining the turgor pressure across the cytoplasmic membrane; and also production of these periplasmic solutes are strongly repressed in hyper-osmotic media [23]. Breedvel and Miller (1994) reported that the maximum level of production of β-glucans occur when cells are grown in hypo-osmotic media [23].

2.1.3.3 Response of symbiotic nitrogen fixation (SNF) to salinity stress

For starting of symbiosis process between soybean and *B. japonicum*, signal molecules should be released and exchanged between them. Salt stress affected more the initial steps of *Bradyrhizobium*-legume symbioses (these steps include the process of signal exchange between legumes and *Bradyrhizobium* bacteria) [167], hence nitrogen fixation. In the presence of salt (NaCl) usually less infection threads are formed in the root hair [167]. Exposure of soybean to salinity stress resulted to decrease in nitrogenase activity, nodule respiration, and nodule oxygen permeability [223]. In addition, Tu (1981) showed that in the presence of 1.0% sodium chloride, soybean root hairs had little curling or deformation when inoculated with *B. japonicum*, and nodulation was completely suppressed by ≥1.2% of NaCl [254].

Moreover Zahran (1999) reported that SNF by legumes is directly related to the salt-induced decline in dry weight and nitrogen content in the shoots [268]. Decreasing the SNF activity by salinity stress is generally attributed to a decline in respiration of the nodules [267,268]. Delgado *et al.* (1994) reported that salt stress decreased in the cytosolic proteins of the nodules, specifically leghemoglobin, and this effect was more significant in pea and bean nodules

than in soybean and faba-bean nodules. Likewise they suggested that salt stress decreases oxygen uptake by the nodules [46].

2.1.3.4 Salinity stress: conclusion

i) Salinization is a major problem in arid and semi-arid lands, and causes poor agricultural productivity.

ii) Decreasing of the soybean yield and the most responses of legumes plants to salinity are such as inhabitation of expansion and curling of root hair, and reducing number of nodules.

iii) Antioxidant defence system of soybean nodules probably responeded to salinity stress.

iv) In hyperosmotic condition *rhizobia* growth is repressed; and *B. japonicum* is salt-sensitive.

v) Under salinity stress *rhizobia* accumulate osmoprotectants, which include some salt ions, carbohydrates, disaccharides, and polysaccharides, e.g. K^+, trehalose, and EPS

vi) In hypo-osmotic conditions, cyclic β-glucans accumulate in the periplasmic space to protect the bacterial cell via decreasing turgor pressure within the cell.

vii) Salinity stress reduces respiration of nodules and leghemoglobin concentration.

2.1.4 Response of SNF to water deficit and desiccation stresses

All living creatures are dependent on water. In nature, plants and microorganisms often encounter water stresses that mainly have a deleterious effect on their life, viability, growth vigour, and productivity. Water deficit stress in soils decreases water content in the roots. Insufficient water availability limits vegetative and reproductive growth of plants [267,268]. Water deficit stress influence growth of *rhizobia* and plants like soybean via two ways: i) by water activity deficiency below a critical tolerance level directly influences *rhizobia* growth; ii) the deficiency causes inferior plant growth, unfavourable the nutrient concentration, undesirable root secreted substances, and unfavourable spatial root growth in turn in soil affects plant productivity and indirectly influences the SNF [267,268].

2.1.4.1 Response of host plants to water deficit stress

Figueiredo *et al.* (1999) and Venkateswarlu *et al*. (1990), reported that tolerance to water deficit stress differs in the species of legume plants and depends on how the plant experiences water deficit stress [62,127,257]. In fact water deficit stress in soil immediately affects turgor pressure of root cells and particularly meristem growth of root. Among negative impacts of water stress are: growth of plant, photosynthates deprivation, undesirable spatial root growth (under drought stress plant roots penetrate deper in soil to find water, which leads to an extreme competition between the growing root and nodulation). There are the most important deleterious effects on symbiotic nitrogen fixing. Meyer and Kouchkovsky (1993) reported that water deficit stress inactivates specifically photosystem II and they suggested loss of active photosystem II centres [163]. In addition light absorption by leaf declined with increasing severity of drought stress [164]. Moreover previous studies reported that water stress decreased relative water content (RWC) of leaves, and later decreased stomatal conductance, which in turn led to decreased CO_2 assimilation, thus increasing the limitation of metabolism likely by limitation of ribulose

bisphosphate (RuBP) synthesis; thus limitation of the RuBP is probably caused by inhibition of adenosine triphosphate (ATP) synthesis, and deficiency of the ATP is likely to result from progressive inactivation or loss of photosystem II centres [128,136,163]

2.1.4.2 Response of *rhizobia* to water deficit stress

Water deficit (commonly known as drought stress) can be defined as lack of sufficient soil water content (moisture) below that which is necessary for a plant to grow normally and complete its life [278,225]. Soil water deficit affects indirectly the niche of root-nodule bacteria, consequently symbiotic nitrogen fixation is significantly sensitive to water deficit stress [127,222,268]. Serraj *et al.* (1999) and Zahran (2010) showed that effects of water stress on SNF have usually been detected as a consequence of direct physiological response acting on nitrogenase activity and involving exclusively one of three mechanisms [222,267]: i) carbon metabolism; ii) oxygen transport into the nodule; iii) feedback on nodule activity by nitrogen compounds are considered in examining nitrogen fixation activity nodule, regulation under conditions of limited water [222], and the most critical period of water stress effect is from attachment and infection stage by *rhizobia* until the functioning of differentiated nodules [222,267]. Therefore, mechanism of symbiotic nitrogen fixing, which mainly includes nodulating, carbon and nitrogen metabolism, nodule O_2 permeability, and particularly nitrogenase activity, is weakened due to water deficit stress.

However, previous studies illustrate that the *rhizobia* could withstand lower water potentials than plant cells and the impact of water deficit stress on the SNF might be due to a direct effect on the microsymbionts [112,222]. As point out earlier species of *rhizobia* differ in tolerance to water deficit stress. For example Zahran (2001) reported that slow-growing species are generally thought to survive desiccation better than fast-growing species [269]. Other studies emphasize that the ecotypes of *rhizobia* strains affect their resistance [278]. For example, native *rhizobia* (when *rhizobia* live as saprophytes, they are generally called native *rhizobia*), or segregated *rhizobia* strains from desert soil

like mesquite *Rhizobium*, had viability for one month, while commercial strains were unable to survive under these conditions [278]. In addition, the distribution and population density of *rhizobia* in soil is affected by initial water content [193]. The previous results illustrate that the water stress response of *rhizobia* strains is morphological changes and continuous water deficit stress will reduce ability of infection to thread and nodulate [29,278].

2.1.4.3 Response of inocula to desiccation stress

Seed inocula storage and promotion of long-term survival are the most important indices for selection of diverse strains of *rhizobia* as well as for commercial purposes. High availability is necessary for their survivial when microsymbionts are inoculated on seeds, root, and carriers (like peat) as a temporary niche. Desiccation is generally defined as lack of water in the bacterial niche. Most of the previous studies suggested that survival of microsymbionts applied to the surface of seeds is poor due to desiccation [28,44,55,258]. Some previous researches showed that the nature of the suspending medium under the recovery of bacteria cells after dehydration is important for the survival of the inocula in the dry state [44,258].

Desiccation stress is enhanced due to other stresses such as: temperature stress, salinity stress, and presence of reactive oxygen species (ROS) during bacteria growth in media, and also due to any agent which decreases water content at the niche of bacterial cells. Previous results [48,258] suggested that the ability of microsymbionts to withstand desiccation stress depends on their ability to cope with all stresses which cause decreased water availability for inocula. Some compounds such as sugars, sugar alcohols, amino acids, polysaccharide, disaccharides and even some minerals like clay have improved ability of microsymbionts to persist desiccation stress [28,44,55,258]. Deaker *et al*. (2007) reported that synthetic polymers such as polyvinyl alcohol (PVA) and polyvinyl chloride (PVC) added to liquid inocula could protect microsymbionts during desiccation stress and they suggested that these polymers probably work via increasing moisture sorption and increase the long-term survival of *rhizobia* [44].

However other researches propose that factors, particularly compounds in the seed coat, could induce the effect of desiccation stress; some seed factors have bilateral effects (reducer and enhancer) on desiccation stress [45,258]. For instance, the existence of polysaccharides and disaccharides in the compounds of the seed's coat (e.g., cellulose, chitin, sucrose, maltose, or trehalose) via hydrophilic properties can help to absorb more moisture and molecules, consequently improve survival desiccation of *rhizobia* [45,153,262].

On the other hand there are some compounds in the seed coat or seed exudates that act as toxins inhibitory to the growth of bacteria (e.g., the derived tannic acid from seed coat display antibacterial ability against 24 standard bacteria strains [264], which is probably a natural defence system for seed storage). For example soybean seed contains substances toxic to the *Bradyrhizobium* [3]. Ali and Loynachan 1990 reported that disrupting the seed coat increased toxicity. In addition the toxicity differs in the species of leguminous seeds as well as different parts of the seed. Also, different strains of *rhizobia* differ in their sensitivity to seed exudates [3].

Tannic acid is a natural defence in some plant seeds, which protects seed against infestation by microorganisms. Ways of influencing the *rhizobia* probably include: i) reducing the bioavailability of some nutrients like iron; ii) forming a protective layer around the seed; iii) deplete protein exudates from *rhizobia* from amino acids. Moreover, previous studies showed that condensed tannins were detected as a main factor in the toxic response of legume seed to *rhizobia* [3,57]. Likewise Prevost *et al.* (1990) reported the suppression of *rhizobia* growth due to tannins [196].

2.1.4.4 Response of SNF to water and desiccation stresses: conclusion

i) Water deficit stress limits growth and development of plant and *rhizobia*, consequently symbiotic nitrogen fixation.

ii) Water deficit stress directly influences *rhizobia* growth, and indirectly, via limitation of vegetative and reproductive growth of plant.

iii) Main responses of plant to water stress are decreasing turgor pressure of root cells, photosynthates deprivation and undesirable spatial root growth.

iv) Water stress affects symbiosis interaction via deleterious carbon storage, oxygen limitation, and nitrogen accumulation in the plant.

v) Slow-growing species could withstand water stress better than species of fast-growing.

vi) Distribution and population density of *rhizobia* in soil is varied due to initial water stress.

vii) Effect of desiccation stress on inocula depend on temperature, salinity and secondary products of metabolite (ROS and RNS) during bacteria growth in medium

viii) Some compounds decrease or remove desiccation stress on inocula e.g., sugars, amino acids, polysaccharides, disaccharides, polymers, and even clay minerals.

ix) Compounds of seed coat have bilateral effects (enhancer and reducer) on desiccation stress.

2.1.5 Response of *rhizobia* to oxidative stress

During the symbiosis between legume and *rhizobia*, in response to infection by *rhizobia*, cellular metabolism root hair cells produce reactive oxygen species (ROS: O_2^-, H_2O_2, $HO^{\cdot}$) which are generated products of the electron transport chain [81,258]. Likewise in response of plant stresses like drought stress (or when biological functions decline due to stresses) these ROS are released and accumulated in cells as deleterious compounds and lead to damaged cells and cell death [185]. The ROS has dual role, so that either they detoxify or destroy cellular macromolecules such as: proteins, lipids, and nucleic acids [48,81].

Thus, bacterial cells perish due to ROS. In addition Deaker *et al.* (2004) showed that decreasing relative humidity (RH< 70%) in the growth field of *rhizobia* caused oxygen molecules to generate their toxic forms and reduced the survival of *rhizobia* in the dry state [45,258]. Production of ROS depends on the rate of aerobic metabolic processes. For example, Becana *et al.* (2000) reported that the increasing rate of respiration in bacteroids and mitochondria caused increased production of ROS [15]. Mostly the ROS are produced in the central zone of bacteroids under severe physiological control with respect to oxygen, and leghemoglobin (Lb) in the nodule is a main source of the ROS [96]. The Lb facilitates oxygen transport to the central zone of bacteroids (zone of nitrogenase activity) at a low concentration but constant flux. In addition other proteins and compounds in the nodule (such as: dinitrogenase (MoFe protein), dinitrogenase reductase (Fe protein), cofactor of nitrogenase, ferrodoxins, xanthine oxidase, uricase, lipoxygenase, rarely catalytic Fe, ascorbate, and thiols) have the capacity bind with oxygen to produce the ROS due to stresses [96].

However, legume nodules have strong strategies to destroy the ROS or prevent their synthesis. Nodules, via formation of antioxidants destroy the ROS. Substances acting as antioxidants include superoxide dismutases (SOD), catalases, ascorbate–glutathione (ASC–GSH) working via cycle, guaiacol and glutathione peroxidase, and ferritin [96]. Mechanisms of action such as: SOD activity to catalyze the dismutation of $O_2^{\cdot-}$ to oxygen; catalases like KatA in *Sinorhizobium meliloti*, or in soybean nodules a typical homotetramer via binding with H_2O_2 removes the hydrogen peroxide generated by uricase; the ASC–GSH cycle by scavenging of H_2O_2 in the cytosol of nodule cells; ferritin by sequestering of catalytic iron; ascorbate and thiol can directly scavenge the ROS; and also mechanism of guaiacol and glutathione peroxidase remove the ROS but the mechanism is still unclear) [96]. Previous studies suggested that the other compounds in the nodules are potent ROS scavengers such as: α-tocopherol (a form of vitamin E) in the soybean nodule, polyamines probably found in nodules, uric acid, and flavonoids and phenolics [96].

Moreover, Herouart *et al.* (2002) reported that the ROS could regulate and be in involved in the expression of plant and/or bacterial genes, which are essential to the nodulation process [100]. Furthermore Vriezen *et al.* (2007) reported that during symbiotic interaction the downregulation of metabolism diminishes production of the ROS [258]. In addition Gibson *et al.* (2008) reported that ROS could support development and growth of infection threads via cross-linking cell wall glycoproteins or degrading cell wall-associated polysaccharides to boost infection thread elongation [81]. It is clear that the *rhizobia* in the early stages of symbiotic interaction are recognized as intruders but based on previous investigations the ROS was not released or accumulated in the cell during the infection stages. Accumulation of the ROS in cells depends on having strains of *rhizobia* compatible with the host root, as well as no stress [15, 96,101]. In fact nodule factors (nodf) (e.g., the LCOs, which are exudations of *rhizobia*) suppress the ROS [96]. Therefore if a strain of *rhizobia* could not be compatible with host root, then defensive response of the legume by the ROS destroy those bacterial cells as intruders.

2.1.5.1 Response of SNF to oxidative stress: conclusion

i) Reactive oxygen species (ROS) are toxic for symbiotic interaction.

ii) Increased ROS under abiotic and biotic stresses are deleterious for symbiotic nitrogen fixation.

iii) ROS have a dual role: the ROS detoxify or destroy cellular macromolecules, depending on rate of metabolic activity as well as stress effect.

iv) Main source of the ROS in nodules is leghemoglobins, in addition other proteins and compounds such as: MoFe protein, Fe protein, cofactor of nitrogenase, ferrodoxins, xanthine oxidase, uricase, lipoxygenase, catalytic Fe, asccorbate and thiols, could produce ROS.

v) Antioxidants destroy the ROS, for example, superoxide dismutases (SOD), catalases, ascorbate–glutathione (ASC–GSH) cycle, guaiacol and glutathione, and ferritin.

vi) In absence of stress, the ROS support development and growth of infection threads.

v) Nodule factors (lipochitooligosaccharides) suppress the ROS, and this response depends on *rhizobial* compatibility with the host root.

2.1.5.2 Response of SNF to reactive nitrogen species (RNS) stress

The RNS are antimicrobial and include mainly nitric oxide ($^{\cdot}NO$), and also $O_2^{\cdot -}$ of the ROS reacts quickly with $^{\cdot}NO$ to form peroxynitrite ($ONOO^-$), then other types of the RNS are produced via reaction of peroxynitrite with other molecules (e.g., $^{\cdot}NO_2$, N_2O_3 and nitrosoperoxycarbonate ($ONOOCO_2^-$)) [84,159]. Previous results reported that RNS and ROS as secondary products of aerobic metabolism in plants and root nodules (bacteroids) arise under abiotic and biotic stresses and extremely disturb normal metabolic activities in all organisms and especially in SNF [84]. In fact nitric oxide (NO) is a lipophilic molecule that diffuses easily through cell membranes. The nitric oxide as signal molecule regulates intracellular and intercellular metabolisms. The nitric oxide has dual effect (positive and negative) in growth and development of bacteria and plants and even animals [84]. Although the role of nitric oxide (NO) in the SNF still needs more evidence, Herouart *et al.* (2002), reported that nitric oxide (NO) forms a complex with leghemogolbin and at low concentration nitric oxide (NO) inhibits nitrogenase acitivity, conversely regulation of oxygen transport to bacteroids promotes enzyme activity for fixing symbiotic nitrogen (positive effect). [100]

In contrast Stohr and Stremiau (2006) suggested that in the presence of a high concentration of nitrate nitrogen in roots by plasma membrane-bound nitrate reductase and nitrite increased nitric oxide (NO) generation, and then high nitric oxide (NO) concentration decelerated the defensive response of the

host plant, deminishing symbiotic interaction nitrogenase activity and even kills plant cells [230]. Further mainly deleterious effects of nitric oxide (NO) are lipid peroxidation, oxidation of tyrosin, *S*-nitrosylation, and also inhibition of enzymes e.g., nitrogenase and cytochrome *c* oxidase [84,100,159,236].

However, there are various molecules such as nitric oxide (NO) scavengers that remove or decrease the deleterious effects of nitric oxide (NO). For example, recently researches showed that hemoglobins could detoxify and modulate nitrite oxide in plants [1,51,157,188]. Perazzoli *et al.* (2004) categorized the kinds of hemoglobins in three classes (symbiotic, non-symbiotic, and truncated) [188]. These molecules scavenge nitric oxide (NO) and reduce nitric oxide (NO) emission under hypoxic stress [157,188]. Furthermore Garcia-Olmedo (2001) and other previous researches reported that deleterious peroxynitrite decreased due to urate (the salt of uric acid) which is a natural peroxynitrite scavenger [1,73,232].

Moreover derived compounds of nitrogen have been identified that cause altered nitrogenase activity. Based on similarly structured molecules, the nitrogenase enzyme has a high capacity to bind with other substrates like NO, NO_3 and NH_3 and even O_2 and cannot discriminate between them [227]. Therefore the quantities of these molecules must be at a low concentration until nitrogenase prefers to bind with N_2 molecules (Table 2-1).

Table 2-1. Substrates of nitrogenase and their products.

Substrates		Products
nitrogen	$N\equiv N$	$NH_3 + NH_3$
acetylene	$HC\equiv CH$	$H_2C=CH_2$
cyanides	$HC\equiv N$, $RC\equiv N$	$CH_4 + NH_3$, $RCH_3 + NH_3$
azides	$H–N^-–N^+\equiv N$	$N_2 + NH_3$
nitrous oxide	$N\equiv N^+–O^-$	$N_2 + H_2O$
isocyanides	$R–N^+\equiv C^-$	$RNH_2 + CH_4$

2.1.5.3 Response of SNF to reactive nitrogen species (RNS): conclusion

i) Generated reactive nitrogen species (RNS) from metabolism in medium, bacteroids and plant are toxic for microsymbionts and plant root cells.

ii) Mostly production of RNS is NO, and in presence of nitrate nitrogen. destroys nitrogenase activity and its effect depend on concentration of NO in the nodule.

iii) Mainly deleterious effects of NO are lipid peroxidation, oxidation of tyrosin, *S*-nitrosylation, and also inhibition of enzymes; e.g., nitrogenase and cytochrome *c* oxidase.

iv) Compounds of hemoglobins scavenge NO in the nodules.

v) Several derived compounds of nitrogen cause diminished nitrogenase activity.

2.2 Magnetite

Iron is the fourth most abundant element in the earth's crust. It exists mainly present in its oxidized state [42]. Plants and microorganisms can uptake iron in its oxidized forms such as ferrous (Fe^{2+}) and ferric (Fe^{3+}). The ferric form is insoluble at neutral and high pH, which make iron unavailable to plants and bacteria in alkaline and calcareous soils [121,126]. Previous studies report that iron has a solubility of 10^{-18} M at biological pH, and solubility further decreased one thousand fold upon each increase in pH by one unit, due to its tendency to form ironhydroxide polymer [35,119,121,213].

Iron is one of important nutrients for plants. Iron is needed for life-sustaining processes from respiration to photosynthesis, where it participates in electron transfer through reversible redox reactions, cycling between Fe^{2+} and Fe^{3+}. Thus, insufficient iron uptake causes chlorosis and yellowing of leave and reduction of plant growth and yield [126].

Plants absorb iron from the soil via two distinct mechanisms (a: reduction-based strategy, b: chelation strategy) according to species [126]. All the dicotyledonous plants (e.g., legume plants) and non-graminaceous monocots (e.g., Arabidopsis) activate the reduction-based strategy (a) when the plants face iron limitation.

The typical (a) response involves the coordinate induction of a core set of three activities (acidification, reduction and transport) at the plasma membrane of root epidermal cells following the onset of iron limitation [126,213]. Acidification of the rhizosphere serves to drive more iron into solution. Investigations suggest that a proton ATPase pumps protons across the plasma membrane in response to iron deficiency [126]. In ferric chelate reduction, iron becomes more available by ferric being reduced to the more soluble ferrous form, and the reduction step, prior to ferrous uptake, has been shown to be critical for iron uptake from iron-deficient soil [126]. In ferrous iron transport, ferrous ions are transported into the root by the iron-regulated transporter protein 1 (IRT1), and this IRT1 is a cytosolic, RNA-binding protein that regulates the translation or stability of mRNAs encoding proteins for iron transport, storage and use. The IRT1 is the major transporter for iron uptake from soil [126].

Strategy b (chelation): in response to iron limitation, grass plants such as corn, wheat and rice secrete compounds known as the mugineic acid family of phytosiderophores, which have a high affinity for ferric ions and efficiently bind them in soil prior to uptake. This strategy is more efficient than the reduction strategy [126,213].

The researches have shown that in the presence of iron, nodulation and nitrogen fixation are increased in legume plants [117,126]. However, Halliwell and Gutteridge (1992) reported that superoxide and hydrogen peroxide (produced in the cells during the reduction of molecular oxygen) are catalyzed by Fe^{2+} and Fe^{3+} to form highly reactive hydroxyl radicals [97], and these

hydroxyl radicals cause damage most cellular components such as DNA, protein, lipids and sugars, but once iron has entered the symplast, it is bound to various chelators, facilitating it remaining in solution and preventing it from participating in the generation of hydroxyl radicals [97,126]. Under iron deficiency, nongrasses plants like legumes uptake iron by releasing protons into the rhizosphere, which cause the pH of the soil to decrease and hence increase the solubility of ferric ions (Fe^{3+}) [126].

However, in order to synthesize iron-containing proteins (e.g., ferredoxin, hyrogenase, cytochromes, nitrogenase, and leghemoglobin), bacteria need to acquire an adequate supply of iron. However taking up the iron at biological pH (pH range of 6 to 8 [261]) is limited for bacteria [93], and under this condition, iron tends to precipitate, forming oxyhydroxide polymers of the general composition FeOOH. For overcoming this problem, bacteria use the siderophores (complex compounds, composed of ferric-specific ligands) and their cognate membrane receptors [92,93]. Bacteria generally can accumulate iron both with self-made siderophores and with siderophores that they themselves do not synthesize [93]. Bacteria species differ in their ability to produce self-made siderophores; for example, in 20 strains of *B. japonicum* only one could produce a siderophore [92].

2.2.1 *Rhizobia* response to iron deficiency

Iron plays a key role in efficient symbiotic nitrogen fixing [34,68,119,198]. The root nodules contain several metalloenzymes and other protein compounds and iron is the main inorganic constituent of them (see section 1.2.1) [126]. Furthermore, *rhizobia* growing under aerobic conditions need iron for a variety of other functions such as reduction of oxygen for synthesis of ATP, reduction of ribotide precursors of DNA, for formation of heme, and for other essential purposes [175,191].

The importance of iron lies in is its redox properties, its potential to elicit damaging free radicals, and also it is the most widely used element in metalloproteins [48]. A bacterial cell contains 10 to 12% of nitrogenase and 25

to 35% leghemoglobin [92,93]. Previous studies illustrate that the iron deficiency may affect the SNF due to impairing *rhizobial* survival, the establishment of functional nodules or the supply of photosynthates and hence energy transfer to the bacteroids [117,184]. Roessler and Nandler (1983) reported that bacterial growth rate and cell viability in the medium decreased when the medium was devoid of iron. In addition iron-deficient cells decreased their cytochrome content and excreted protoporphyrin into the growth medium [211].

Previous studies show that a concentration of at least one micromolar iron is needed for optimum growth of microoganisms [175]. Under iron deficiency *rhizobia* like *B. japonicum* generally produce siderophores, which are able to scavenge free iron in the niche of *rhizobial* cells, and they can compete effectively with hydroxyl ion for the ferric state of iron [129,175,191]. In fact the siderophores are ferric ion chelating agents with relatively low molecular weight and elaborated by bacteria [175,191]. In other word siderophores are ferric ion-specific ligands with high affinity for iron that are taken into the cell via specific membrane receptors [191]. Their role is to scavenge iron from the environment and to make the mineral in a form available to the bacteria for growth [175,191].

However, *B. japonicum* bacteroids in soybean nodules can import soluble iron ions (ferrous), which are produced probably by a nodule-specific ferric-reductase [33, 48,129]. Moreover, Kaiser *et al*. (2003) reported that *B. japonicum* has a protein similar to the Feo (ferrous iron transport), which is used for ferrous ion import [121]. Moreover, *rhizobia* have several iron-transport pathways such as using ferric citrate (but the mechanism is still unknown), using heme and heme-protein complex as a source of Fe, and directly Fe^{3+} [33,48,129].

2.2.2 Plant's response to magnetite nanoparticles

Engineered magnetite nanoparticles display fascinating physico-chemical properties that lead to their use for the improvement of biological systems, promotion of innovative biotechnology, and the design of new biodiagnostics [118,147,151,154]. To date nanoparticles in agriculture are used mainly as

fertilizers, fungicides and disinfectants for enhancing plant growth, but there are contradictory results in respect to their effects [26,151,114,137,143]. Nair *et al*. (2010) reported that delivery of magnetic nanoparticles in plants and during growth was not toxic [173]. Lu *et al.* (2002) reported that nitrate reductase in soybean increased due to a mixture of nano-SiO_2 and nano-TiO_2 as well as enhancement of its water and nutrients use efficiency [151], in addition it possibles hastened seed growth [142]. Hong *et al*. (2005) showed that when spinach was treated with 0.25 % nano-TiO_2 enhanced the photosynthesis efficiency and the nitrogen metabolism, and suggested that the reason may be activation of the photochemical reaction of chloroplasts of spinach [106]. Yang and Watt (2005) reported that root elongation of plant crops was reduced slightly by alumina nanoparticles [265]. Zhu *et al*. (2008) reported that pumpkin (*Cucurbita maxima*) plants could grow in 0.5 g L^{-1} magnetite nanoparticles they had no toxicological effects on plants [275]. Doshi *et al.* (2008) illustrated that in presence of a high on concentration of nano aluminium (up to 17 mg L^{-1}) in water irrigation; California red kidney bean (*Phaseolus vulgaris*) did not uptake aluminium, whereas ryegrass showed accumulated aluminium in its leaf [52]. Coating nanoparticles with natural organic might promote their positive effects. Liu *et al.* (2008) showed that coating magnetite nanoparticles with humic acid remove toxic heavy metals such as Hg (II), Cd (II), and Cu (II) from water [146].

However, other researches show that applied nanoparticles could be toxic and/or induce oxidative stress in some plants. Wang *et al*. (2010) reported that magnetite nanoparticles induced oxidative stress in ryegrass and pumpkin plants [260]. Lin and Xing (2008) reported that the total dry matter of ryegrass plant was reduced due to using ZnO nanoparticles in the field [141].

Moreover, nanomaterials products constitute a new type of pollution in ecosystems. To date for solving this problem many researches provide the basis for serious legislations that prevent adding toxic nanomaterials to the environment. For instance, the community health and consumer protection agency of the European Commission reported that the toxicity of nanoparticles is reduced or possible removed if they are fixed in a matrix [2,59].

2.2.3 Effect of magnetite nanobiocomposites on bacteria

Magnetite nanobiocomposites are composed of magnetite nanoparticles embedded in an amorphous matrix of natural polymers [116]. Many researchers have tried to investigate the interaction between the coating polymer and the Fe_3O_4 nanoparticles. Non-toxic and biocompatible nature are the main criteria for the selection of polymers for fabrication of magnetite nanobiocomposites for biological systems [249].

Magnetite nanobiocomposites represent a class of functional materials that may have potential for use in bacterial growth, protection of cell wall, and enhancing cell viability [138,251]. To ensure of their survival in adverse conditions (toxic substances, pH, temperature, oxygen reactive potential, and electrical conductivity), bacteria change their behaviour to adapt to the new conditions, although this chang may damage some bacterial cells [249]. Clearly the bacterial cell membrane is main responsible for exchanging materials and if destroyed means the end of bacterial life.

For this purpose carriers have been used as substances that protect bacterial cells on inoculated seeds during storage and/or in soil until the infection step into the host root, e.g., peat and natural polymers). Jung and Mugnier (1982) reported that application of polymers (e.g., alginate and xanthan) can increase survival and cell viability as inoculants [120]. Introducing effective *rhizobia* to the soil and subsequently the rhizosphere of legumes depends on the composition and type of carriers in the preparation of inoculants [228].

Previous studies illustrated that up to 25% of yield in crop legumes depends on the successful application of inoculants [45]. To be uneconomical by applying higher quantities of microsymbionts (for supplying of fresh inoculants, some countries like Iran have to buy every year) required the use of polymers in order to preserve inoculants in storage or on seed. Deaker *et al.*'s (2004) criteria for polymers required to protect the survival of *rhizobial* cells on seed include: non-toxic and free from preservatives that may be harmful to

rhizobial cells [45]; dispersable in water to permit release of cells from the polymer matrix; no competition for using water in niche of the *rhizobial* cell, and also it at least can remove and/or diminish seed coat toxicity [45].

Mostly to date polymeric adhesives such as methylcellulose, gum arabic, polyvinylacetate, polyvinylpyrrolidone, and caseinate salts are recommended in preparation of inoculants [45]. The main reason for inoculation is to provide sufficient number of viable effective microsymbionts to induce rapid colonisation of the rhizophere allowing nodulation to take place quickly after seed germination and gain optimum yield [45,228]. Deaker *et al.* (2004) reported that the applied polymers in the inoculation encapsulate *rhizobial* cells, and also viability of *B. japonicum* varied in several polymers [45]. Mugnier and Jung (1985) illustrated that the viability seem to be more directly related to some properties of the water in biopolymers. In fact they described that by entrapping of *rhizobial* cells the biopolymers contribute properties such as ability to limit heat transfer, high water activity and good rheological properties [169].

However, there is no direct research regarding application of bio-nanobiocomposites (e.g., magnetite nanobiocomposites) as adhesive polymers in the preparation of inoculation. But there are bio-nanobiocomposites which seem that they could play an effective role for the protection of bacterial cell wall under environmental stress [105,192]. In addition some nanomaterials via their branching and their connectors can connect to either to the bacterial cell walls and play a role as a protection layer for cell and/or as scavengers bind to toxic substances, and as a result decrease impacts of stresses [192]. Hereby, increasing the number of branches of nanoparticles can increase the probability of attachment to the bacterial cell wall.

One of strategies in this study was to simultaneously apply magnetite nanoparticles and natural polymers as bio-nanobiocomposites and we supposed that this new type of magnetite bio-nanocomposite can decrease effectively the decay time of the bacterial cell wall via scavenging of the ROS and/or maybe the RNS in the medium and also remove and/or diminish the effect of toxic compounds which are in the seed coat (Figure 2-1).

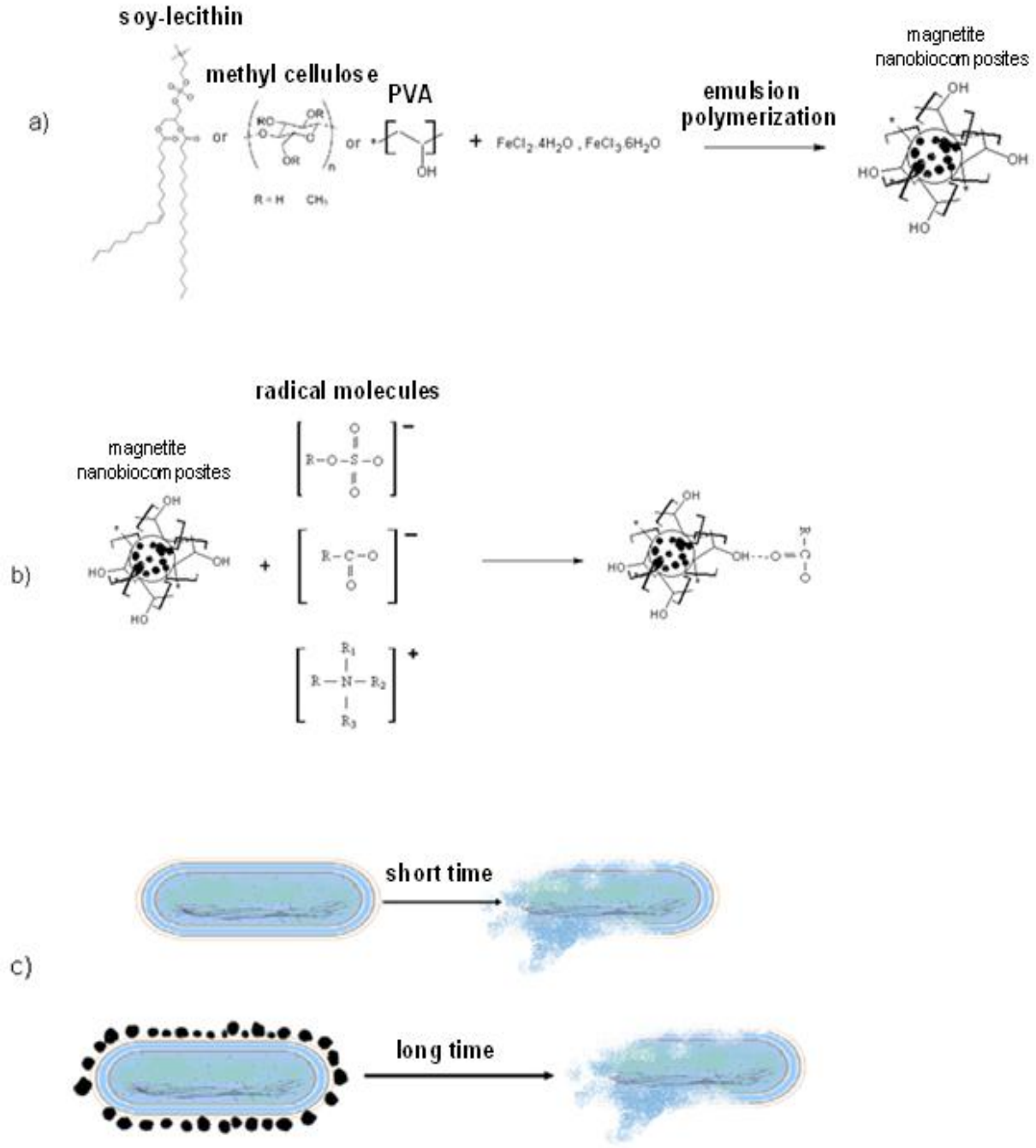


Figure 2-1. Schematic shows fabrication of magnetite nanoparticles with some polymers (a), to bind nanobiocomposites with the ROS (b), and attachment of nanobiocomposites on the bacterial cell wall for increasing survival (c).

2.2.4 Magnetite: conclusions:

i) Plants and microorganisms can uptake iron in its oxidized forms such as ferrous (Fe^{2+}) and ferric (Fe^{3+}). At neutral and high pH the ferric form is insoluble and is unavailable to plants and bacteria.

ii) Iron is the most important constituent of protein compounds in the root nodule for successful SNF.

iii) The most important aspects of application of magnetite nanoparticles in SNF are the redox properties and the ability of damaging free radicals in the niche of bacterial culture.

iv) Siderophore compounds are an effective tool for reducing the impact of iron deficiency.

v) Nanoparticles play an important role in improving existing crop management techniques, for instance using nanoparticles could improve plants growth in soybean and spinach (although there are other reports which show negative responses of these nanoparticles).

vi) To date many efforts expose some ways to prevent toxicity of nanoparticles, e.g., to fix nanoparticles in a matrix.

vii) Fabrication of magnetite nanobiocomposites by using natural polymers could be useful to increase use of natural polymers for protection of bacterial cell wall and viability.

viii) Natural polymers are generally used as carriers and protectors of *rhizobial* cells during storage and in soil against decomposers.

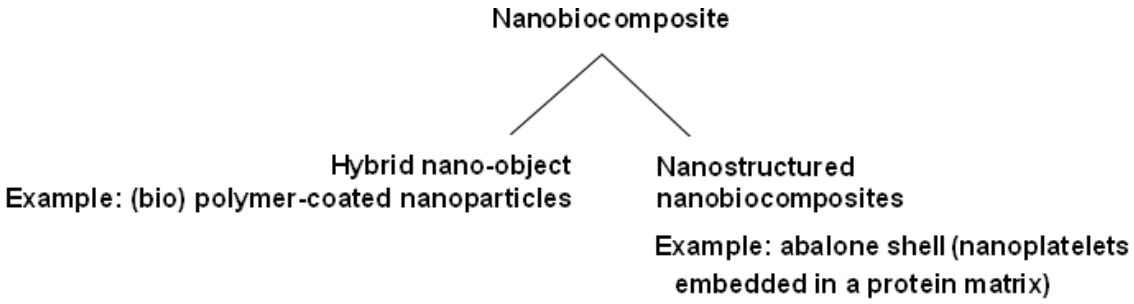
ix) The criteria of polymers to use in the preparation of inocula for legume seed include non-toxic and free from preservatives, dispersable in water, not competing for water content with *rhizobial* cells, and removal of seed coat toxicity.

x) The polymer can entrap *rhizobial* cells and limit heat transfer, maintain high water activity and maintain good rheological properties.

ix) Using combination capacities of magnetite nanoparticles and natural polymers in order to promote carrier defence potential was an opportunity to develop utilization of magnetite nanobiocomposites.

2.3 Terminology

The general term “nanobiocomposite” is used by Pomogailo [192] to describe composites containing biological and nonbiological nanomaterial components. The composite can be either a discrete object such as a particle or a structured bulk material. We have the following hierarchy:



For example, in section 3.1.3 we describe nanobiocomposites, which are constructed from magnetite practicle coated with a polymer. However, in this study the word “nanobiocomposite” is used in order to describe magnetite nanoparticles coated by polymers like polyvinylalcohol (PVA) and/or natural polymers like soylecithin or methylcellulose (which are actually hybrid nano-object).

3 Materials and Methods

3.1 Nanomaterials

3.1.1 Fabrication of magnetite nanoparticles

A co-precipitation method is used to achieve the nanoparticles nearly of uniform size and shape [6]. A 0.5 M solution of sodium hydroxide (about 125 ml) was poured in to a three necked flask under nitrogen gas with vigorous stirring at 65 ºC. 12.5 ml of an equimolar mixture of iron (II) and iron (III) (counter ion = Cl^-) (each 0.9 M) was prepared in deaerated distilled water and further purged with nitrogen gas for 30 min [79]. Then the iron solution was added dropwise to the sodium hydroxide during 30 min while stirring vigorously. The suspensions were separated and purified by centrifuging and resuspending three times in water and then HCl at 20,000 *g*. The particles were finally dried in a vacuum oven at 70-80 ºC [79].

3.1.2 Nanoparticle characterization

Transmission electron microscopy (TEM) (model: Philips XL30) was used to characterize the particles. The final nanoparticle slurry was sonicated for approximately for 5 min to better disperse the nanoparticles; a drop was placed on a carbon-coated copper TEM grid (200–300 mesh) and then left to dry in air. The particle diameters were determined directly from the TEM images and had a mean value of 17 nm (Figures 3-1, 5-3 and B).

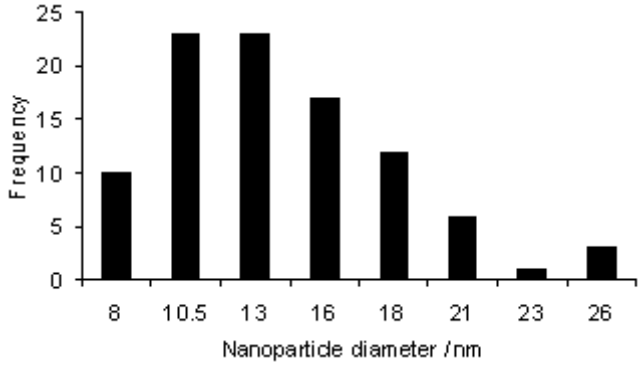


Figure 3-1. Bar chart of the particle sizes from a typical preparation determined from TEM images (100 particles were measured).

3.1.3 Fabrication of nanobiocomposite

Following the literature review (section 2.2.3) soylecithin, methylcellulose and polyvinyl alcohol (PVA) were selected as polymers. This fabrication was the same as for the pure magnetite nanoparticles except that for PVA (20%), soylecithin (10%) or methylcellulose (10%) to the iron solution prior to sodium hydroxide addition.

3.1.4 Nanobiocomposite characterization

The prepared nanobiocomposites were characterized by the following methods as discussed below.

3.1.4.1 Fourier transform infrared (FTIR)

To provide evidence for grafting of polymers and nanoparticles, FTIR spectra of native polymers and nanoparticles were recorded from 4,000 to 400 cm^{-1} using a Thermo Nicolet Avatar 370 FTIR spectrophotometer. Transmission IR spectra of thin films prepared by the powder casting method were recorded. The prepared films were directly mounted in the spectrometer.

3.1.4.2 X-ray diffraction (XRD)

Characteristics of physical magnetite nanoparticles such as crystalline particles, amorphous particles, size, and also discover performance of

biopolymers (e.g., when a biopolymer like soylecithin is used for coating nanoparticles) in order to promote and adjust the nanoparticles' properties, is a necessary condition, especially when they will used in liquid culture media and symbiotic nitrogen fixation. Thus, effectiveness of using magnetite nanoparticles depends on the discovery and development of their positive characteristics. However, the change of nanoparticles' properties like size due to different methods of fabrication, brings about change of XRD diffraction patterns, then the size can be estimated by using of Scherrer formula.

The XRD studies of the nanoparticles and nanobiocomposites were carried out on a Siemens D5005 X-ray powder diffractometer in a standard Bragg-Brentano geometry. The diffraction was collected from 5 to 90°, 2°θ with a step size of 0.05°. The average crystallite size of iron oxide particles were estimated using Scherrer's formula, and the average crystallite size (*d*) is determined from the following equation [10]:

$$d = \frac{k\lambda}{\beta cos\ \theta} \qquad (3\text{-}1)$$

where d is mean grain size, k is the shape factor (0.9), β is broading of the diffraction angle and θ is the Bragg angle, and λ is diffraction wavelength (1.54 A˚) [10].

3.2 Bacterial strain and culture media

A particular strain, called Histic, of *Bradyrhizobium* japonicum was obtained from the Soil and Water Institute, Tehran, and majority farmers in the south west of Iran use of the *B. japonicum*, therefore in this study firstly has focussed on the species. For preparation of the bacterial culture 50 ml of yeast mannitol broth (YMB) (Table 3-1) in a 250 ml flask was autoclaved (121 °C, 15-20 min, 103 kPa), and after cooling the bacteria were introduced to the flask. This mother culture was then kept in a shaking incubator (150 rpm) at 28-30 °C for 8-10 days. In order to check for contaminants, a loop was streaked on glucose peptone agar (GPA) solid medium (on which a majority of all bacteria are able

to grow except *Bradyrhizobium*), which was then kept in an incubator at 28-30 ºC for 24 h. 1 ml of contamination-free mother culture (with a population of 10^9 cells per ml) was removed and sequentially repeatedly tenfold diluted in a series of test tubes containing 9 ml of distilled water to achieve a final population of 10^4 viable cells per ml. 1 ml from each dilution was transferred to the new 250 ml flasks again containing 50 ml YMB that had been autoclaved as previously [228,79].

Table 3-1. Constituents of yeast mannitol broth (YMB) as a liquid media and yeast mannitol agar (YMA) as a solid media [180,64].

Media	Material	Amount / g l^{-1}
YMB	K_2HPO_4	0.5
	$MgSO_4$	0.2
	NaCl	0.1
	Yeast extract	0.5
	Mannitol	10
YMA	YMB	1 litre
	Agar	15-18 g

Note that these are complex media. No iron was expressly added, and none or a negligible quantity was present in the yeast extract.

3.2.1 Transmission electron micrograph (TEM) of bacteria

TEM was also used to visualize *Bradyrhizobium japonicum* Histic. 1.5 ml of the bacterial culture was removed after 6 days growth and thrice centrifuged at 3,000 *g* for 5-7 min followed by resuspending in ultrapure water. One drop of the supernatant was then placed on the grid and left overnight at room temperature to dry before examing in the TEM.

3.2.2 Pour-plate count

The pour-plate count method is considered one of the most reliable and accurate methods for measuring the number of viable cells per ml [43]. The procedure is as follows: after inoculation of the culture flasks, 1 ml from each flask was removed separately and eight times sequentially tenfold diluted in new test tubes. To count the bacteria, twice 100 µl from each of these eight tubes was removed and spread on two 9 cm Petri dishes containing YMA solid

medium (Table 3-1) incorporating Congo Red indicator to verify the absence of invasive foreign strains during incubation. It is convenient if there are between 30 and 300 cells on the Petri dishes. The Petri dishes were immediately transferred to an incubator and kept at 28-30 ºC for 6-8 days. Counting the number of colony-forming units N_f on the surface of the Petri dishes was started after 5-6 days [228,79]. Measurement of medium pH was carried out simultaneously for all treatments at 7 days using a glass electrode.

3.3 Growth curve analysis

Growth of bacteria reproducing by binary fission can be plotted as the logarithm of the number of viable cells N_V versus the incubation time t, and the evolution typically has four distinct phases (Figure 3-2) [194], which are as follows:

Lag Phase: no increase in cell number occurs, cell division does not take place and there is no net increase in mass, the cell is synthesizing new components.

Log Phase: Bacteria are growing and dividing exponentially at the maximal rate, characterized by a growth rate constant.

Stationary Phase: population growth ceases. This phase is usually attained by bacteria at a population level of around 10^9 cells per ml.

Death Phase: decline in N_V due to detrimental environmental changes like nutrient deprivation and the buildup of toxic waste.

N_V was determined from the following equation [180]:

$$N_V = N_C \times D_S \qquad (3\text{-}2)$$

where N_V is the number of viable cells per ml, N_C is the number of colonies on the plate, and D_S is reciprocal of dilution of sample.

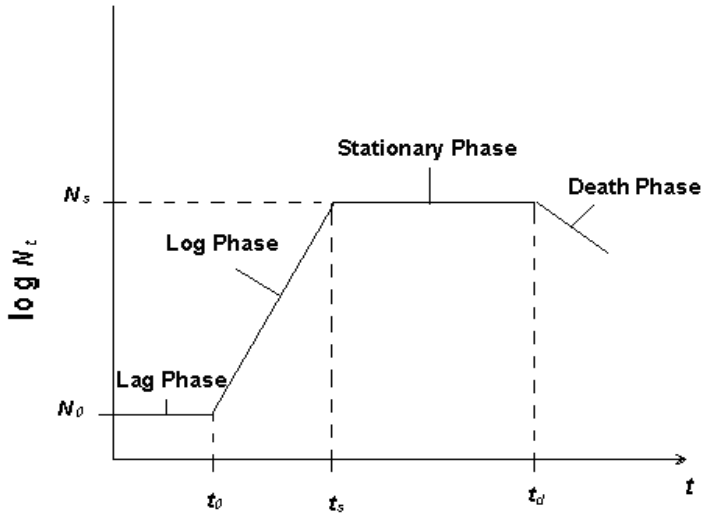


Figure 3-2. Typical stages of bacterial growth [79].

3.3.1 Growth rate constant (GRC)

During the logarithmic phase, the bacterial culture mimics a first-order chemical reaction; i.e., the rate of increase of cells is proportional to the number of bacteria present at that time. The GRC is determined from the following equation [194,79];

$$\ln N_s - \ln N_0 = \mathrm{GRC}(t_s - t_0) \qquad (3\text{-}3)$$

where N_0 is the number of viable cells at t_0 (see Figure 3-1), the time at the end of the lag phase, and N_s is the number of viable cells at the end of the log phase (time t_s). However, increase the GRC is positive response in bacteria growth.

3.3.2 Number of generations (NG)

NG is an index of bacterial growth useful for comparison of strains during the logarithmic phase growing under different conditions, and increase the NG is a positive response during bacteria growth. This index is calculated from the following equation [194,79]:

$$\mathrm{NG} = (\log_{10} N_s - \log_{10} N_0)/ \log_{10} 2 \qquad (3\text{-}4)$$

3.3.3 Mean generation time (MGT)

The mean generation time (MGT) is calculated from the following equation [194,79]:

$$MGT = (t_s - t_0)/NG \quad (3\text{-}5)$$

However, decrease the MGT is a positive response for bacterial species.

3.4 Bacterial survival on seeds

Seeds of soybean *Glycine max* L. Merr (cultivar L.504) (this line was member of maturity groups 6 and 7, and its maturity time was about 160 to 180 days) were obtained from the Safi Abad Agriculture Research Centre, Dezfoul, Iran. The bacterial culture was described as above (Section 3.2) except that at the stationary phase (with a population of 10^9 viable cells ml^{-1}) 250 µl of the media (which were mixed with different concentrations of magnetite nanoparticles) was applied to each of 250±1 seeds of soybean and glass beads (4 or 5 mm). Seeds and glass beads were surface-sterilized through treating with 95% alcohol for 10 s to remove waxy material, then with sodium hypochlorite (2.5% commercial bleach) in which the seeds were immersed for 3-5 min, after which the bleach was drained off and the seeds washed with at least six changes of sterile water [228]. The seeds and the glass beads were grouped to two sets. Each set had six sterilized 250 ml flasks, and each flask contained 20 of the seeds or the glass beads separately. 125 µl of the liquid Gum Arabic (40%) was added to each flask, which then gently shaken by hand for 1 min. Seeds were then dried on clean paper for 3-5 min. Later each flask was inoculated with 20 µl of liquid media and it was slowly shaken by hand for 1 min and seeds again dried on clean paper for 3-5 min at room temperature. When the seeds and the glass beads appeared dry, they were placed in flasks were kept at 25 ºC or 4 ºC for 9 days. This work was repeated for each concentration of magnetite nanoparticles [228,79].

3.5 Water activity (a_w)

Bacterial growth and division depend on the water activity (a_w) [19]. The amount of water available to the bacteria can be reduced by interaction with solute molecules (the solvation effect) or by adsorption to the surfaces of chaotropic solids (chaotrope) [22]. The water activity is the ratio of the vapour pressure p of water in a material (liquid medium) to the vapour pressure p_0 of pure water at the same temperature [14], i.e.:

$a_w = p/p_0$ (3-6)

The a_w was measured by using an Aqualab Water Activity meter. 5 ml of the liquid medium were placed in a 10 ml disposable cup placed, then placed in the meter. The sample was sealed by a lid (to determine a_w unit ± 0.003 accuracy). After 3 to 5 min, vapour was equilibrated. The average of three determinations was recorded. This variable was measured at the beginning of the experiment (before inoculation) and 7 days after inoculation.

3.6 Oxidation reactive potential (ORP)

The oxidation reaction potential (ORP) was determined by detecting the concentration ratio of a selected ion in the reduced form to the same in the oxidized form. An ORP electrode measures the redox potential according to the Nernst half-cell potential equation [6]. The ORP was measured at the beginning of the experiment (before inoculation) and 7 days after inoculation.

3.7 Viability of inoculants

This variable is number of the viable cells ml^{-1} on the seeds and glass beads [180]. The bacteria viability measured immediately after inoculation of the seeds and glass beads (SG) (Figure 3-3). Seeds and glass beads were separately grouped in flasks equal to the number of treatments, so that each of flask was contained of 200 seeds or glass beads. Of each flask was taken 20 seeds or glass beads and they divided into four sub groups of the seeds or the

glass beads in test tubes (each tube was contained of 5 seeds or 5 glass beads). Then these tubes were contained of distilled water (10 mm), after vortexing (402 *g*) for 2 min, one ml from each tube was removed and six times sequentially fivefold diluted in new test tubes. To count the bacteria, twice 100 µl of each of these six tubes was removed and spread on two 9 cm Petri dishes containing YMA solid medium incorporating Congo Red indicator to verify the absence of invasive foreign strains and Brilliant Green indicator will suppress fungal growth during incubation. The Petri dishes were immediately transferred to the incubator and kept at 28-30 ºC for 6-8 days. Counting the number of colony forming unit N_f on the surface of the Petri dishes was started after 5-6 days [228,78].

Figure 3-3. Inoculation of the *Bradyrhizobium* cells on the seeds and glass beads.

3.8 Soybean cultivation

The inoculated seeds were planted in pots which contained of sterilized horticultural sand. First the sand was washed in a container with a water tap, then immersed in 0.5 % HCl for 1-2 days, then the acid was drained off, and then the sand was washes with at least 6-8 changes sterile water. If needed 1 M KOH was used to adjust the pH, since it should be 6.8 ± 2. 1.5 kg of the sand was placed in each pot. Dimensions of each pot were upper diameter 19 cm, lower diameter 13.3 cm, and height 17 cm. The pots were then autoclaved for 4-6 h at 121 °C and 131 kPa, after cooling the inoculated seeds were planted in the pots. Three seeds were planted (2.5-3.5 cm in depth) in each pot, after the

seeds emerged only one seedling was kept in each pot. The pots were then placed in the greenhouse with 22-25 °C day / 15-18 °C night, and with ambient light. Each pot was irrigated with 500 ml of tap water every day. In order to prevent intereference from nutrient effects on nodulation, no nutrient was used in the medium, and no Fe source was added (hence inducing iron deficient conditions).

3.9 Biochemical determinations

3.9.1 Isoflavonoid extraction and identification

After 45-60 days planting, roots of soybean were cut off, weighed, immediately frozen in liquid nitrogen, and finely ground. The powder was mixed with HPLC-grade methanol, using 2 ml of methanol for 100 mg of powder weight tissue. After vigorous shaking and centrifugation for 10 min at 7,000 rpm, the supernatant was removed and evaporated under a nitrogen stream, and the remaining substance redissolved in HPLC-grade methanol at 100 μl g^{-1} of tissue. The root extract genistein was separated on HPLC system (Kontron 5XX) with a C-18 column (Vydac, 250 mm × 4.6 mm) with the following solvents: A=ultrapure water containing 0.09% trifluoroacetic acid; B=acetonitrile containing 0.09% trifluoroacetic acid. The column was eluted isocratically with solvent A for 5 min and a linear gradient of 0 to 100% B between 5 and 25 min at ambient temperature with a flow rate of 1 ml min^{-1} [160].

3.9.2 Isolation and purification of lipochitooligosaccharide (LCO)

The two litre of *Bradyrhizobium* culture (the culture method is described in section 3.2) was extracted with 40% HPLC-grade 1-butanol by shaking the mixture for 5-10 min and then allowing the two phases to separate for 24 h. The organic phase (butanol layer) was collected and evaporated at 80 °C in a rotary evaporator (Figure 3-4). The final volume, 2-3 ml, was dissolved in 4 ml of 18% of acetonitrile and stored in the dark in glass tubes, at 4 °C for 24 h. Samples were centrifuged for 10 min at 1200 *g*, and the supernatant was collected for HPLC analysis [197].

For analytical purposes, 200μl of the nodf extract were injected into the HPLC system (Kontron 5XX) at 214 nm. Separation was carried out with a Vydac C-18 reversed-phase column (5 μm, Vydac, 250 mm×4.6 mm). To elute nodf from the column, a programme of acetonitrile and water gradients was used: 18% acetonitrile (10 min), a linear gradient from 18% to 60% acetonitrile (20 min), and a linear gradient from 60% to 100% acetonitrile, and a linear gradient from 100% to 18% acetonitrile (5 min), and finally 18% acetonitrile for 5 min. The peak with a retention time of 38.5-40.70 min was identified as Nod Bj-V ($C_{18:1}$, MeFuc) by comparing its retention time with standard nodf [197].

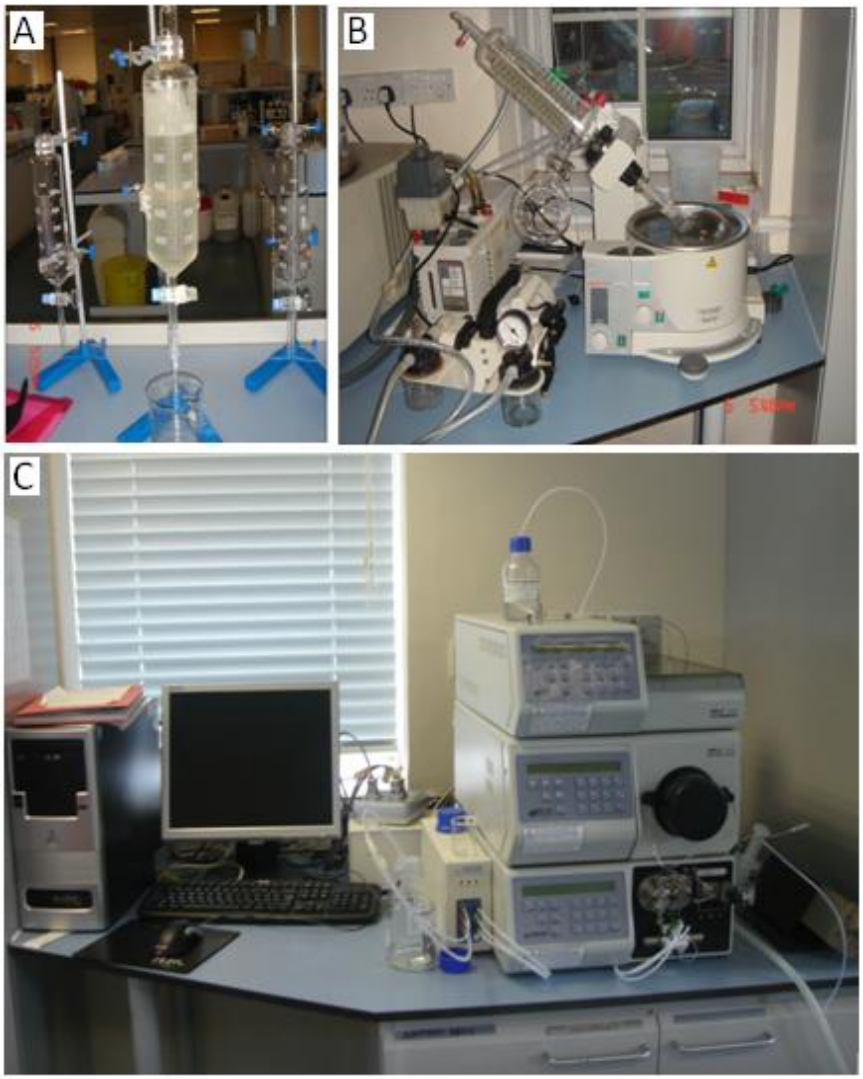


Figure 3-4. *Bradyrhizobium* culture provided in seprator (A) for translocation to rotary evaporator (B), then indentification of the LCO by HPLC instrument (C).

3.10 Measurement of dry weight of the vegetative components

After 60 to 70 days of planting date, the vegetative components of soybean grown in the green house such as root, stem, leaves separately cut, and were weighed (fresh weight), then dried in an oven at 70 to 80˚C for 48 hours and again weighed (dry weight) (Figure 3-5).

Figure 3-5. The vegetative components of soybean grown in the oven.

3.11 Counting of the branches and nodes on the main stem

At harvest time (60 to 70 days after planting) the number of nodes on the main stem, and also the number of branches, were deterrmined.

3.12 Measurement of the plant height

At harvest time the height was determined by ruler (cm) from the base of the main stem to the upmost tip of the leaf (Figure 3-6).

Figure 3-6. Measurement of the plant height. The upmost tip is at the very top of the picture.

3.13 Acetylene reduction assay (ARA) for measuring nitrogense activity of root nodules

The ARA is an indicator of N_2 fixation activity [52]. After 60-70 days from planting soybean in the greenhouse, the upper 5 cm section of nodulated roots (plant collar) were cut and placed in 300 ml glass bottles, and the bottles were sealed with a rubber stopper. 10% of the air in the bottle was removed with a vacuum pump and the same volume of C_2H_2 was injected into the bottles. The bottles were incubated for 1 h at 25-30 °C. C_2H_4 produced was measured by GC 8000 series, Carlo Erba Instruments. After being assayed, nodules were detached from the roots, weighed, and counted. C_2H_2 reduction activity was calculated as nmol of generated C_2H_4 (mg nodules)$^{-1}$ h^{-1}, generated C_2H_4 (mg nodule)$^{-1}$ h^{-1} or generated C_2H_4 (single nodule)$^{-1}$ h^{-1}. Endogenous C_2H_4 (e.g., produced from cut sections of plant) was taken into account by strictly carrying out the same procedure for every experiment.

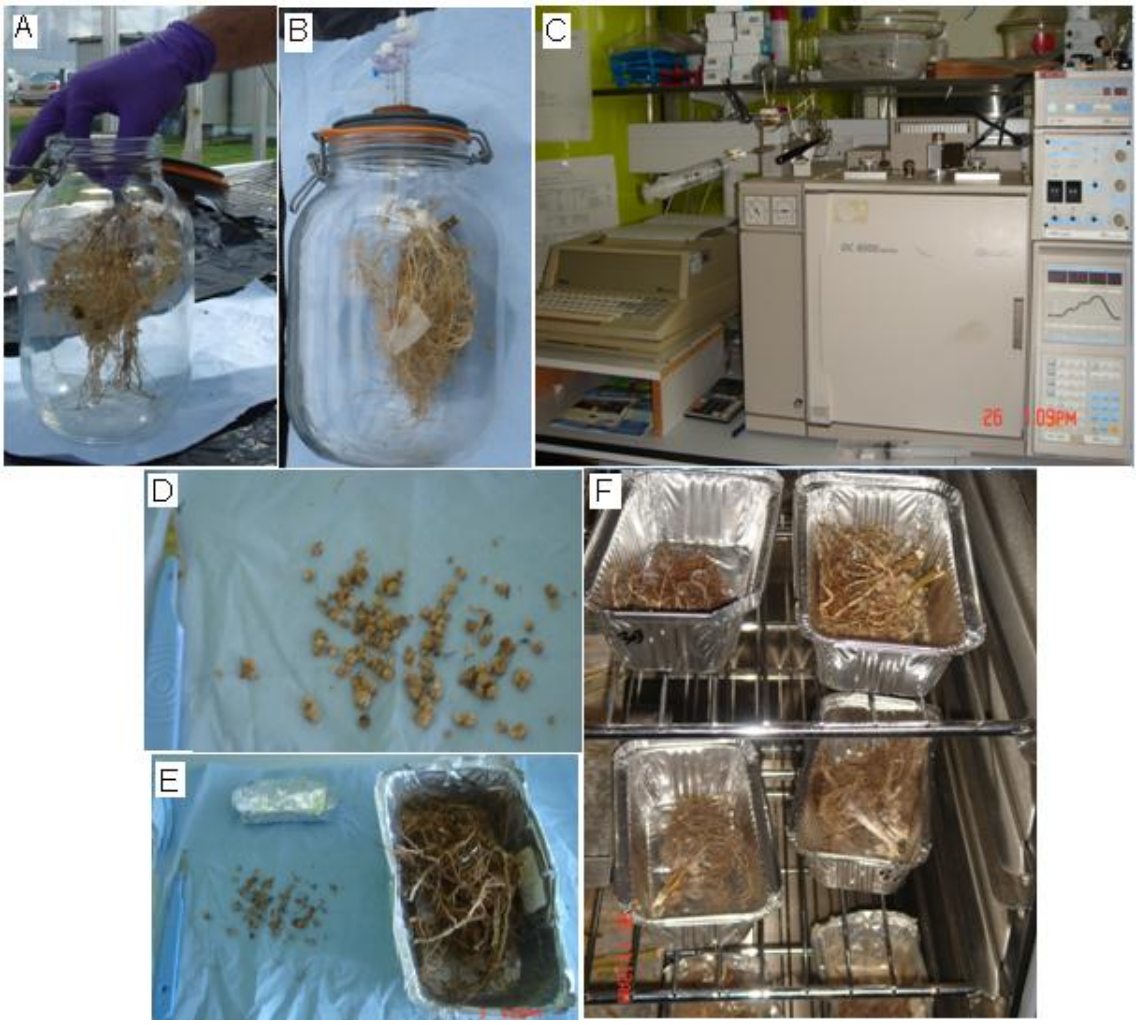


Figure 3-7. Process of measuring nitrogen generated from nodules: (A), cut root and put in a bottle with volume 1-3 litre; (B) injection of C_2H_2 in the bottle; (C), measuring generated ethylene by GC instrument; (D and E), detach nodules from root and weigh; (F), dry the nodules and root in an oven.

3.14 Statistical methods

3.14.1 Randomized block design (RBD)

In this study the RBD was used for some experiments. RBD is the standard design for agricultural experiments. The blocking technique is used to account for possible heterogeneity in experimental circumstances. The experimental “field “(e.g., shaking incubator, greenhouse) is divided into blocks. Each block is divided into plots (e.g., culture flasks, petri dishes, pots). Treatments are then assigned at random to plots in the blocks—one replication

of each treatment in each block; that is to say any treatment can be adjacent to any other treatment, but not to the same treatment within the block. The number of blocks equals the number of replications; if effects are additive, the greater the number of replications, the less the uncertainty of the mean of observations. The statistical model for the RBD can be written in several ways. The traditional model is an effects model [107,168], so that:

$$y_{nk} = \mu + \tau_n + \beta_k + \epsilon_{nk} \qquad (3\text{-}7)$$

Table 3.2 gives the meaning of the symbols.

Table 3-2. Definition of statistical parameters in a RBD

t is the number of treatments
r is the number of blocks
n (1,2, ... , t) is the treatment number
k (1,2, ... , r) is the block number
y_{nk} is observed data (e.g., plant height) from each plot
μ is overall mean
τ_n is the effect of the *n*th treatment
β_k is the effect of the *k*th block
ϵ_{nk} is the random error of the observation
y_{no} is the total of all observations taken under treatment n
y_{oo} is the grand total of all observations
y_{ok} is the total of all observations in block k
N is the total number of observations
SS_{Total} is sum of squares of the grand total of all observations
$SS_{\text{Treatments}}$ is sum of squares of the total of all observations taken under treatment n
SS_{Blocks} is sum of square of the total of all observations in replication k
SS_E is sum of square of the random error of all the observations
MS is mean sum of squares which often abbreviated as mean squares.
$MS_{\text{Treatments}}$ is mean squares of treatments
MS_{Blocks} is mean squares of replications
MS_E is mean squares of error
F test is ratio of variance of treatments to variance of error, and ratio variance of blocks to variance of error.

Figure 3.8 shows a schematic example of the treatment assigned to plots.

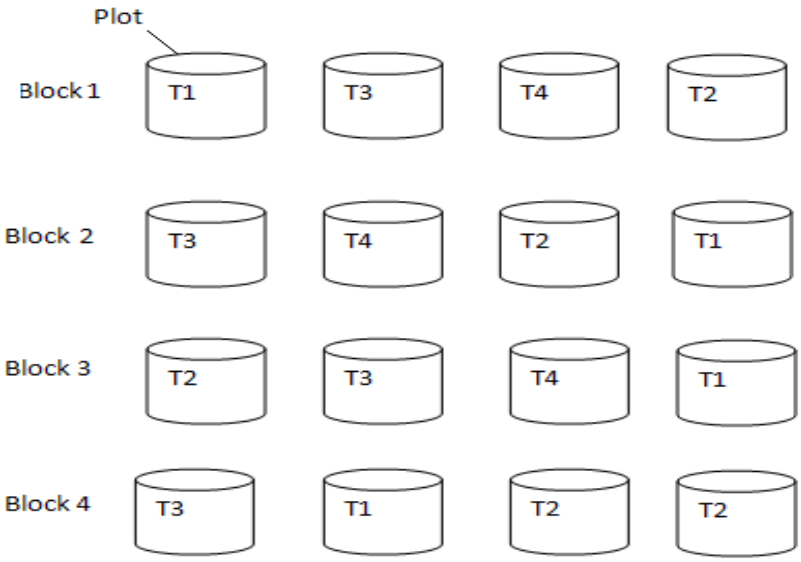


Figure 3-8. Schematic of an example of the RBD.
This experiment has four treatments (T1, T2, T3, T4) and four blocks. Randomization was done in Microsoft Excel.

The calculations of the analysis of variation table of the RBD are as follows:

$$SS_{\text{Treatments}} = \frac{\sum_{n=1}^{t} y_{io}^2}{r} - \frac{y_{oo}^2}{N} \quad (3\text{-}8)$$

$$SS_{\text{Blocks}} = \frac{\sum_{k=1}^{r} y_{ok}^2}{t} - \frac{y_{oo}^2}{N} \quad (3\text{-}9)$$

$$SS_{\text{Total}} = \sum_{n=1}^{t} \sum_{k=1}^{r} y_{nk}^2 - \frac{y_{oo}^2}{N} \quad (3\text{-}10)$$

$$SS_E = SS_{\text{Total}} - SS_{\text{Treatments}} - SS_{\text{Blocks}} \quad (3\text{-}11)$$

The analysis of variation table format of the RBD is given in table 3.3.

Table 3-3. Analysis of variation table for the RBD with a single factor.

Source of variation (SOV)	Degrees of freedom (*df*)	Sum of squares (*SS*)	Mean squares (*MS*)	*F* test
Blocks	$(r-1)$	SS_{Blocks}	$MS_{Blocks} = SS_{Blocks} / (r-1)$	MS_{Blocks} / MS_{E}
Treatments	$(t-1)$	$SS_{Treatments}$	$MS_{Treatments} = SS_{Treatments} / (t-1)$	$MS_{Treatments} / MS_{E}$
Error (or Residual)	$(r-1)(t-1)$	$SS_{E} = SS_{Total} - SS_{Treatments} - SS_{Blocks}$	$MS_{Er} = SS_{E} / (r-1)(t-1$	
Total	$N-1 = (r \times t) - 1$	SS_{Total}		

3.14.2 Two ways factorial arrangement on a RBD

For experiments which involve two factors (e.g., nanoparticle concentration and different pH) two way factorial arrangement on a RBD was used (see Figure 3.9). Treatments of factor levels were combined. They were arranged within a randomized block design (RBD), assigning of the treatments was the same as one way design (see section 3.12.1). There are several ways to write the model for a factorial experiment. The effects model [197,168] is:

$$y_{ijk} = \mu + A_i + B_j + AB_{ij} + \beta_k + \epsilon_{ijk} \qquad (3\text{-}12)$$

Table 3.4 gives the meaning of the symbols.

Table 3-4. Definition of statistical parameters in a two way factorial arrangement on a RBD.

k (1,2, ... , r) is the block number
i (1,2, ... , *a*) is the number of the levels of factor *A*
j (1,2, ..., *b*) is the number of the levels of factor *B*
y_{ijk} is observation or data (e.g., plant height) of each plot
μ is overall mean
A_i is the effect of the *i*th level of (treatment) factor *A*
B_j is the effect of the *j*th level of (treatment) factor *B*
ϵ_{ijk} is the random error of the observation
y_{ook} is the total of all observations taken under blocks r
y_{ioo} is the total of all observation taken under factor *A*
y_{ojo} is the total of all observations taken under factor *B*
$y_{ooo}$is the grand total of all observations
N= abr is the total number of observations
SS_{Blocks} is sum of squares of the total of all observations taken under blocks *r*
SS_{Total} is sum of squares of the grand total of all observations
SS_A is sum of squares of the total of all observations taken under factor *A*
SS_B is sum of squares of the total of all observations in factor *B*
SS_{AB} is sum of squares of the total of all observations under between factors *A* and *B*
SS_E is sum of square of the random error of all the observations
MS is mean sum of squares which often abbreviated as mean squares.
MS_{Blocks} is mean squares of blocks r
MS_A is mean squares of factor *A*
MS_B is mean squares of factor *B*
MS_{AB} is mean squares of interaction of factors *AB*
MS_E is mean squares of error
F test is ratio of variance of factor *A* to variance of error, ratio variance of factor *B* to variance of error, and ratio variance of interaction of factors(*AB*) to variance of error

Figure 3.9 shows that a schematic example of the treatment assigned to plots.

Figure 3-9. Schematic of a factorial experiment with two factors, A and B. The first factor has three level of treatments (a1, a2, a3), and the second factor has two (b1, b2). Note that the assignment of particular combination of treatments to plots is random. Randomization was done in Microsoft Excel.

The calculations of a factorial experiment with two factors, A and B are as follows:

$$SS_A = \frac{\sum_{i=1}^{a} y_{ioo}^2}{br} - \frac{y_{ooo}^2}{N} \quad (3\text{-}13)$$

$$SS_B = \frac{\sum_{j=1}^{b} y_{ojo}^2}{ar} - \frac{y_{ooo}^2}{N} \quad (3\text{-}14)$$

$$SS_{AB} = \frac{\sum_{i=1}^{a}\sum_{j=1}^{b} y_{ijo}^2}{r} - \frac{y_{ooo}^2}{N} - SS_A - SS_B \quad (3\text{-}15)$$

$$SS_{\text{Blocks}} = \frac{\sum_{k=1}^{r} y_{ook}^2}{ab} - \frac{y_{ooo}^2}{N} \quad (3\text{-}16)$$

$$SS_{\text{Total}} = \sum_{i=1}^{a}\sum_{j=1}^{b}\sum_{k=1}^{r} y_{ijk}^2 - \frac{y_{ooo}^2}{N} \quad (3\text{-}17)$$

$$SS_E = SS_{\text{Total}} - SS_A - SS_B - SS_{AB} - SS_{\text{Blocks}} \quad (3\text{-}18)$$

The analysis of variation table format of the two way factorial arrangement on a RBD is given in table 3.5.

Table 3-5. Analysis of variation table for the two way factorial arrangement on a RBD, when number of factor are two with different levels (3×2).

Sources of variation (SOV)	Degrees of freedom (*df*)	Sum of squares (*SS*)	Mean squares (*MS*)	*F* test
Blocks	$(r-1)$	SS_{Blocks}	$MS_{\text{Blocks}} = SS_{\text{Blocks}} / (r-1)$	$MS_{\text{Blocks}} / MS_E$
A	(*a*-1)	SS_A	$MS_A = SS_A / (a\text{-}1)$	MS_A / MS_E
B	(*b*-1)	SS_B	$MS_B = SS_B / (b\text{-}1)$	MS_B / MS_E
AB	(*a*-1)(*b*-1)	SS_{AB}	$MS_{AB} = SS_{AB} / (a\text{-}1)(b\text{-}1)$	MS_{AB} / MS_E
Error (or Residual)	(*ab*-1)$(r-1)$	$SS_E = SS_{\text{Total}} - SS_A - SS_B - SS_{AB} - SS_{\text{Blocks}}$	$MS_E = SS_E / (ab\text{-}1)(r-1)$	
Total	(*abr*-1) or (*N*-1)	SS_{Total}		

3.14.3 Four ways factorial arrangement on a RBD

In this study a four way factorial arrangement on a RBD was used, and similar by the two factor experiments all treatments combining factors were arranged within on a RBD. Assigning of the treatments was the same as in one way design (see section 3.12.1). There are several ways to write the model for a four way factorial experiment. The effects model is [197,168]:

$$y_{ijglk} = \mu + A_i + B_j + C_g + D_l + AB_{ij} + AC_{ig} + AD_{il} + BC_{jg} + BD_{jl} + CD_{gl} + ABC_{ijg} + ABD_{ijl} + BCD_{jgl} + ACD_{igl} + ABCD_{ijgl} + \beta_k + \epsilon_{ijglk} \quad (3\text{-}19)$$

Table 3.6 gives the meaning of the symbols.

Table 3-6. Definition of statistical parameters in the four way factorial arrangement on a RBD.

k (1,2, ... , r) is the block number
i (1,2, ... , a) is the number of the levels of factor *A*
j (1,2, ..., b) is the number of the levels of factor *B*
g (1,2, ... , c) is the number of the levels of factor *C*
l (1,2, ..., d) is the number of the levels of factor *D*
y_{ijglk} is observation or data (e.g., plant height) of each plot
μ is an overall mean
A_i is the effect of the *i*th factor *A*
B_j is the effect of the *j*th factor *B*
C_g is the effect of the gth factor *C*
D_l is the effect of the lth factor *D*
r_k is the effect of the kth block r
ϵ_{ijglk} is the random error of the observation
y_{ioooo} is the total of all observations taken under factor *A*
y_{ojoo} is the total of all observations under factor *B*
y_{oogoo} is the total of all observations under factor *C*
y_{ooolo} is the total of all observations under factor *D*
y_{ooook} is the total of all observations under blocks r
y_{oooo} is the grand total of all observations
y_{ijglo} is the total of all observations under all factors (*ABCD*)
N is the total number of observations (*abcdr*)
SS_{Blocks} is sum of squares of the grand total of all observations under blocks *r*
SS_{Total} is sum of squares of the grand total of all observations
SS_A is sum of squares of the total of all observations taken under factor A
SS_B is sum of squares of the total of all observations in factor B
SS_C is sum of squares of the total of all observations in factor C
SS_D is sum of squares of the total of all observations in factor D
SS_{AD} is sum of squares of the total of all observations under between factors A and D
SS_{AC} is sum of squares of the total of all observations under between factors A and C
SS_{AD} is sum of squares of the total of all observations under between factors $A$and D
SS_{BC} is sum of squares of the total of all observations under between factors B and C
SS_{BD} is sum of squares of the total of all observations under between factors B and D
SS_{CD} is under between factors $C$and D
SS_{ABC} is sum of squares of the total of all observations under between factors A, B, and C

SS_{ABD} is sum of squares of the total of all observations under between factors A, B, and D
SS_{BCD} is sum of squares of the total of all observations under between factors B , C, and D
SS_{ADC} is sum of squares of the total of all observations under between factors A, D, and C
SS_{ABCD} is sum of squares of the total of all observations under between factors A, B, C, and D
SS_E is sum of square of the random error of all the observations
MS is mean sum of squares which often abbreviated as mean squares.
MS_{Blocks} is mean squares of blocks r
MS_A is mean squares of factor *A*
MS_B is mean squares of factor *B*
MS_{AB} is mean squares of interaction of factors (*AB*)
MS_{AC} is mean squares of interaction of factors (*AC*)
MS_{AD} is mean squares of interaction of factors (*AD*)
MS_{BC} is mean squares of interaction of factors (*BC*)
MS_{BD} is mean squares of interaction of factors (*BD*)
MS_{CD} is mean squares of interaction of factors (*CD*)
MS_{ABC} is mean squares of interaction of factors (*ABC*)
MS_{ABD} is mean squares of interaction of factors (*ABD*)
MS_{BCD} is mean squares of interaction of factors (*BCD*)
MS_{ACD} is mean squares of interaction of factors (*ACD*)
MS_{ABCD} is mean squares of interaction of factors (*ABCD*)
MS_E is mean squares of errors
F test is ratio of variance of factor *A* to variance of error, ratio variance of factor *B* to variance of error, ratio variance of factor *C* to variance of error, ratio variance of factor *D* to variance of error, ratio variance of interaction of factors(*AB*) to variance of error, ratio variance of interaction of factors(*AC*) to variance of error, ratio variance of interaction of factors(*AD*) to variance of error, ratio variance of interaction of factors(*BC*) to variance of error, ratio variance of interaction of factors(*BD*) to variance of error, ratio variance of interaction of factors(*CD*) to variance of error, ratio variance of interaction of factors(*ABC*) to variance of error, ratio variance of interaction of factors(*ABD*) to variance of error, ratio variance of interaction of factors(*BCD*) to variance of error, ratio variance of interaction of factors(*ACD*) to variance of error, and ratio variance of interaction of factors(*ABCD*) to variance of error.

For example in an experiment, first factor with three levels (a_1, a_2, a_3), second factor with two levels (b_1, b_2), third factor with two levels (C_1, C_2), and fourth factor with two levels (d_1, d_2), then treatments are as follows:

(**a1b1c1d1, a1 b_1c1d2, a1 b_1c2d1, a_1b1c2d2, a1b_2c1d1,a1b2c1d2, a1b2c2d1, a1b2c2d2, a2b1c1d1, a2b1c1d2, a2b1c2d1, a2b1c2d2, a2b2c1d1, a2b2c1d2, a2b2c2d1, a2b2c2d2, a3b1c1d1, a3b1c1d2, a3b1c2d1, a3b1c2d2, a3b2c1d1, a3b2c1d2, a3b2c2d1, a3b2c2d2**).

Figure 3-10 shows that a schematic example of the treatment assigned to plots. Each block involves 24 plots (Figure 3-10).

Block 1 (Replication)	Block 2 (Replication)	Block 3 (Replication)	Block 4 (Replication)
a1b1c1d1	a2b2c1d1	a1b2c2d1	a2b2c1d2
a1 b1c1d2	a2b2c1d2	a1b2c2d2	a2b2c2d1
a1 b1c2d1	a2b2c2d1	a2b1c1d1	a2b2c2d2
a1b1c2d2	a2b2c2d2	a2b1c1d2	a3b1c1d1
a1b2c1d1	a3b1c1d1	a2b1c2d1	a1b1c1d1
a1b2c1d2	a3b1c1d2	a2b1c2d2	a1 b1c1d2
a1b2c2d1	a3b1c2d1	a2b2c1d1	a1 b1c2d1
a1b2c2d2	a3b1c2d2	a2b2c1d2	a1b1c2d2
a2b1c1d1	a3b2c1d1	a2b2c2d1	a1b2c1d1
a2b1c1d2	a3b2c1d2	a2b2c2d2	a1b2c1d2
a2b1c2d1	a3b2c2d1	a3b1c1d1	a3b1c1d2
a2b1c2d2	a3b2c2d2	a1b1c1d1	a3b1c2d1
a2b2c1d1	a1b1c1d1	a1 b1c1d2	a3b1c2d2
a2b2c1d2	a1 b1c1d2	a1 b1c2d1	a3b2c1d1
a2b2c2d1	a1 b1c2d1	a1b1c2d2	a3b2c1d2
a2b2c2d2	a1b1c2d2	a1b2c1d1	a3b2c2d1
a3b1c1d1	a1b2c1d1	a1b2c1d2	a3b2c2d2
a3b1c1d2	a1b2c1d2	a3b1c1d2	a1b2c2d1
a3b1c2d1	a1b2c2d1	a3b1c2d1	a1b2c2d2
a3b1c2d2	a1b2c2d2	a3b1c2d2	a2b1c1d1
a3b2c1d1	a2b1c1d1	a3b2c1d1	a2b1c1d2
a3b2c1d2	a2b1c1d2	a3b2c1d2	a2b1c2d1
a3b2c2d1	a2b1c2d1	a3b2c2d1	a2b1c2d2
a3b2c2d2	a2b1c2d2	a3b2c2d2	a2b2c1d1

Figure 3-10. Schematic example of the four way factorial arrangement on a BRD, the treatments combined of factors were assigned into plots. Note that the assignment of particular combination of treatments to plots is random. Randomization was done by in Microsoft Excel.

The calculations of a factorial experiment with four factors, A, B, C, and D are as follows:

$$SS_A = \frac{\sum_{i=1}^{a} y_{ioooo}^2}{bcdr} - \frac{y_{oooooo}^2}{N} \quad (3\text{-}20)$$

$$SS_B = \frac{\sum_{j=1}^{b} y_{ojooo}^2}{acdr} - \frac{y_{oooooo}^2}{N} \quad (3\text{-}21)$$

$$SS_C = \frac{\sum_{g=1}^{c} y_{oogoo}^2}{abdr} - \frac{y_{oooooo}^2}{N} \quad (3\text{-}22)$$

$$SS_D = \frac{\sum_{l=1}^{d} y_{ooolo}^2}{abcr} - \frac{y_{oooooo}^2}{N} \quad (3\text{-}23)$$

$$SS_{AB} = \frac{\sum_{i=1}^{a}\sum_{j=1}^{b} y_{ijooo}^2}{cdr} - \frac{y_{oooooo}^2}{N} - SS_A - SS_B \quad (3\text{-}24)$$

$$SS_{AC} = \frac{\sum_{i=1}^{a}\sum_{g=1}^{c} y_{iogoo}^2}{bdr} - \frac{y_{oooooo}^2}{N} - SS_A - SS_C \quad (3\text{-}25)$$

$$SS_{BC} = \frac{\sum_{j=1}^{b}\sum_{g=1}^{c} y_{ojgoo}^2}{adr} - \frac{y_{oooooo}^2}{N} - SS_B - SS_C \quad (3\text{-}26)$$

$$SS_{AD} = \frac{\sum_{i=1}^{a}\sum_{l=1}^{d} y_{ioolo}^2}{bcr} - \frac{y_{oooooo}^2}{N} - SS_A - SS_D \quad (3\text{-}27)$$

$$SS_{BD} = \frac{\sum_{j=1}^{b}\sum_{l=1}^{d} y_{ojoolo}^2}{acr} - \frac{y_{oooooo}^2}{N} - SS_B - SS_D \quad (3\text{-}28)$$

$$SS_{CD} = \frac{\sum_{g=1}^{c}\sum_{l=1}^{d} y_{oooglo}^2}{abr} - \frac{y_{oooooo}^2}{N} - SS_C - SS_D \quad (3\text{-}29)$$

$$SS_{ABC} = \frac{\sum_{i=1}^{a}\sum_{j=1}^{b}\sum_{g=1}^{c} y_{ijgoo}^2}{dr} - \frac{y_{oooooo}^2}{N} - SS_A - SS_B - SS_C \quad (3\text{-}30)$$

$$SS_{ABD} = \frac{\sum_{i=1}^{a}\sum_{j=1}^{b}\sum_{l=1}^{d} y_{ijolo}^2}{cr} - \frac{y_{oooooo}^2}{N} - SS_A - SS_B - SS_D \quad (3\text{-}31)$$

$$SS_{BCD} = \frac{\sum_{j=1}^{b}\sum_{g=1}^{c}\sum_{l=1}^{d} y_{ojglo}^2}{ar} - \frac{y_{oooooo}^2}{N} - SS_B - SS_C - SS_D \quad (3\text{-}32)$$

$$SS_{ACD} = \frac{\sum_{i=1}^{a}\sum_{g=1}^{c}\sum_{l=1}^{d} y_{ioglo}^2}{br} - \frac{y_{oooooo}^2}{N} - SS_A - SS_C - SS_D \quad (3\text{-}33)$$

$$SS_{ABCD} = \frac{\sum_{i=1}^{a}\sum_{j=1}^{b}\sum_{g=1}^{c}\sum_{l=1}^{d} y_{ijglo}^2}{r} - SS_A - SS_B - SS_C - SS_D - SS_{AB} - SS_{AC} - SS_{AD} - SS_{BC} - SS_{BD} - SS_{CD} - SS_{ABC} - SS_{ABD} - SS_{BCD} - SS_{ACD}$$ (3-34)

$$SS_{\text{Blocks}} = \frac{\sum_{k=1}^{r} y_{ooook}^2}{abcd} - \frac{y_{ooooo}^2}{N}$$ (3-35)

$$SS_E = SS_{\text{Total}} - SS_A - SS_B - SS_C - SS_D - SS_{AB} - SS_{AC} - SS_{AD} - SS_{BC} - SS_{BD} - SS_{CD} - SS_{ABC} - SS_{ABD} - SS_{BCD} - SS_{ACD} - SS_{ABCD} - ss_{\text{Blocks}}$$ (3-36)

$$SS_{\text{Total}} = \sum_{i=1}^{a}\sum_{j=1}^{b}\sum_{g=1}^{c}\sum_{l=1}^{d}\sum_{k=1}^{r} y_{ijglk}^2 - \frac{y_{ooooo}^2}{N}$$ (3-37)

The analysis of variation of the four way factorial arrangement results on a RBD given in table 3.7.

Table 3-7. Analysis of variation for the four way factorial arrangement on a RCB.

Source of variation (SOV)	Degrees of freedom (*df*)	Sums of squares (*SS*)	Mean squares (*MS*)	*F* test
Blocks	$(r-1)$	SS_{Blocks}	$MS_{\text{Blocks}} = SS_{\text{Blocks}} / (r-1)$	$MS_{\text{Blocks}} / MS_E$
Factor *A*	(*a*-1)	SS_A	$MS_A = SS_A / (a\text{-}1)$	MS_A / MS_E
Factor *B*	(*b*-1)	SS_B	$MS_B = SS_B / (b\text{-}1)$	MS_B / MS_E
Factor *C*	(*c*-1)	SS_C	$MS_C = SS_C / (c\text{-}1)$	MS_C / MS_E
Factor *D*	(*d*-1)	SS_D	$MS_D = SS_D / (d\text{-}1)$	MS_D / MS_E
AB	(*a*-1)(*b*-1)	SS_{AB}	$MS_{AB} = SS_{AB} / (a\text{-}1)(b\text{-}1)$	MS_{AB} / MS_E
AC	(*a*-1)(*c*-1)	SS_{AC}	$MS_{AC} = SS_{AC} / (a\text{-}1)(c\text{-}1)$	MS_{AC} / MS_E
AD	(*a*-1)(*d*-1)	SS_{AD}	$MS_{AD} = SS_{AD} / (a\text{-}1)(d\text{-}1)$	MS_{AD} / MS_E
BC	(*b*-1)(*c*-1)	SS_{BC}	$MS_{AB} = SS_{BC} / (b\text{-}1)(c\text{-}1)$	MS_{BC} / MS_E
BD	(*b*-1)(*d*-1)	SS_{BD}	$MS_{AB} = SS_{BD} / (b\text{-}1)(d\text{-}1)$	MS_{BD} / MS_E
CD	(*c*-1)(*d*-1)	SS_{CD}	$MS_{AB} = SS_{CD} / (c\text{-}1)(d\text{-}1)$	MS_{CD} / MS_E
ABC	(*a*-1)(*b*-1)(*c*-1)	SS_{ABC}	$MS_{ABC} = SS_{ABC} / (a\text{-}1)(b\text{-}1)(c\text{-}1)$	MS_{ABC} / MS_E
ABD	(*a*-1)(*b*-1)(*d*-1)	SS_{ABD}	$MS_{ABD} = SS_{ABD} / (a\text{-}1)(b\text{-}1)(d\text{-}1)$	MS_{ABD} / MS_E
BCD	(*b*-1)(*c*-1)(*d*-1)	SS_{BCD}	$MS_{BCD} = SS_{BCD} / (b\text{-}1)(c\text{-}1)(d\text{-}1)$	MS_{BCD} / MS_E
ABC	(*a*-1)(*c*-1)(*d*-1)	SS_{ACD}	$MS_{ABC} = SS_{ACD} / (a\text{-}1)(c\text{-}1)(d\text{-}1)$	MS_{ACD} / MS_E
ABCD	(*a*-1)(*b*-1)(*c*-1)(*d*-1)	SS_{ABCD}	$MS_{ABCD} = SS_{ABCD} / (a\text{-}1)(b\text{-}1)(c\text{-}1)(d\text{-}1)$	MS_{ABCD} / MS_E
Error (or Residual)	(*abcd*-1)$(r-1)$	$SS_E = SS_{\text{Total}} - SS_A - SS_B - SS_C - SS_D - SS_{AB} - SS_{AC} - SS_{AD} - SS_{BC} - SS_{BD} - SS_{CD} - SS_{ABC} - SS_{ABD} - SS_{ACD} - SS_{BCD} - SS_{ABCD} - SS_{\text{Blocks}}$	$MS_E = SS_E / (abcd\text{-}1)(r\text{-}1)$	
Total	(*abcdr*-1) or (*N*-1)	SS_{Total}		

3.14.4 Least significant difference (LSD) test

For comparing treatment means with control, when hypothesis of equal means has been rejected by using the *F* test (see Table 3.6), the least significant diffirence (LSD) was used in some experiments. If the number of replications in all treatments were equal, the calculation of LSD is [197,168]:

$$LSD = \sqrt{\frac{2MS_E}{r}} \times t_{(\frac{\alpha}{2},\ df_e)} \quad (3\text{-}38)$$

where MS_E is the mean squares of error (residual), which is obtained from analysis of variation table, r is number of replications, $t_{(\frac{\alpha}{2})}, df_e$ are obtained from a standard table of student's t distribution with degrees of freedom (df_e) and probability level ($\frac{\alpha}{2}$) [197, 168]. The same, arbitrarily assigned, alphabetic letter lables a group of means (upper case for Alpha=0.05, lower case for Alpha=0.01) that are not significantly different from each other (identified using LSD); different letters to lable means that are significantly different. However, if the *F* test was not significant this method should not be used (as there are no significant differences between the means)

All calculations were carried out using MSTATC software and were plotted by Microsoft Excel software.

4 Experimental design

4.1 Bradyrhizobium growth rate (BGR)

4.1.1 Effects of magnetite nanoparticles on *Bradyrhizobium* growth rate (BGR)

The treatments with nanoparticles are given in Table 4-1. The magnetite nanoparticles were added to the liquid medium before autoclaving. The statistical design was randomized block design (RBD) with four blocks. The most important variables were growth rate coefficient (GRC), mean generation time (MGT), and number of generations (NG) of bacteria before the stationary phase.

Table 4-1. Definition of the magnetite treatments.

Treatment designation	Concentration of nanoparticles / µg ml^{-1}
C_1	0
C_2	20
C_3	40
C_4	60
C_5	80

4.1.1 Effects of pH and magnetite nanoparticles on *Bradyrhizobium* growth rate (BGR)

Treatments with nanoparticles and pH are designated in Table 4-2. The magnetite nanoparticles were added to the liquid medium (YMB) and the pH adjusted by adding 1 M NaOH or HCl before autoclaving. Statistical design was two way factorial arrangement (3×6) on a randomized block design (RBD) with four blocks, the first factor being the different concentrations of nanoparticles and the second factor the different pH values. The most important variables were bacterial growth rate (BGR), growth rate constant (GRC), mean

generation time (MGT), number of generations (NG) of bacteria before the death phase, oxidation reaction potential (ORP), and water activity (a_w).

Table 4-2. Definition of the magnetite treatments.

Treatments designation	Levels
First Factor: Magnetite nanoparticle concentration (N_P) µg ml^{-1}	0, 40, 80
Second factor: pH	4, 5, 6, 7, 8, 9

4.1.2 Effects of salinity and magnetite nanoparticle concentration on *Bradyrhizobium* growth rate (BGR)

This experiment was carried out in two groups of magnetite nanoparticle concentration.

4.1.2.1 Effects of magnetite nanoparticle concentration (0, 40, 80 µg ml^{-1}) and salinity (0, 600, 1200, ..., 4800 µS cm^{-1}) on *Bradyrhizobium* growth rate (BGR)

Treatments with nanoparticles and salinity are designated in Table 4-3. The magnetite nanoparticles were added to the liquid medium (YMB) and the salinity was calculated according to the relation of total dissolved salts (TDS) to electrical conductivity (EC) and then by adding NaCl (powder) to the YMB before autoclaving. The salinity is determined from the following equation [224]:

$$TDS(\mathrm{mg\,l^{-1}}) = EC(\mu\mathrm{S\,cm^{-1}}) \times 0.6 \qquad (4\text{-}1)$$

The statistical design was two way factorial arrangement (3×9) on a randomized block design (RBD) with four blocks, the first factor being the different concentrations of nanoparticles and the second factor the different pH values. The most important variables were bacterial growth rate (BGR), growth rate constant (GRC), mean generation time (MGT), number of generations (NG)

of bacteria before the death phase, oxidation reaction potential (ORP), and water activity (a_w).

Table 4-3. Definition of the magnetite treatments.

Treatments designation	Levels
First Factor: Magnetite nanoparticle concentration (N_P) / µg ml^{-1}	0, 40, 80
Second factor: Salinity (S) / µS cm^{-1}	0, 600, 1200, 1800, 2400, 3000, 3600, 4200, 4800

4.1.2.2 Effects of magnetite nanoparticle concentration (0, 20, 60 µg ml^{-1}) and salinity (0, 600, 1200, ..., 4800 µS cm^{-1}) on *Bradyrhizobium* growth rate (BGR)

Treatments with nanoparticles and salinity are designated in Table 4-4. The set up was in section 4.1.2.1.

Table 4-4. Definition of the magnetite treatments.

Treatments designation	Levels
First Factor: Magnetite nanoparticle concentration (N_P) / µg ml^{-1}	0, 20, 60
Second factor: Salinity (S) / µS cm^{-1}	0, 600, 1200, 1800, 2400, 3000, 3600, 4200, 4800

4.2 Effects of desiccation, temperature, seed and magnetite nanoparticles effects on *Bradyrhizobium japonicum* viability as inocula

Treatments are given in Table 4-5. Statistical design was four way factorial arrangement (6×2×2×6) on a randomized block design (RBD) with four blocks. The most important variable was cell viability of the *Bradyrhizobium* on the surface of seeds and glass beads.

Table 4-5. Definition of treatments.

Treatments designation	Levels
First factor: Desiccation times	0, 24, 48, 72, 96, 216
Second factor: Temperature (T ºC)	25 and 4
Third factor: Seeds (SG)	Soybean seeds and Glass beads
Fourth factor: Magnetite nanoparticle concentration (N_P) / µg ml^{-1}	0, 20, 40, 60, 80, 100

4.3 Secretion

4.3.1 Effect of magnetite nanoparticles concentration on excretion of lipochitooligosaccharide (LCO) from *Bradyrhizobium japonicum*

The treatments with nanoparticles are given in Table 4-6. The magnetite nanoparticles were added to the liquid medium before autoclaving. The statistical design was randomized block design (RBD) with four blocks. The variable measured was quantity of secreted LCO from bacterial cells before the stationary phase.

Table 4-6. Definition of the magnetite treatments.

Treatment designation	Concentration of nanoparticles / µg ml^{-1}
C_1	0
C_2	20
C_3	40
C_4	60
C_5	80
C_6	100

4.3.2 Effect of coating *Bradyrhizobium japonicum* with magnetite nanoparticles on secreted genistein from soybean roots

The treatments with nanoparticles are given in the Table 4-7. The magnetite nanoparticles were added to the liquid medium before autoclaving. Before stationary phase seeds were inoculated with 1-2 µl $seed^{-1}$ of liquid medium containing 10^6 or 10^7 *B. japonicum* ml^{-1} liquid medium based on treatments. The statistical design was randomized block design (RBD) with four blocks. The variable measured was quantity of secreted genistein from soybean roots 45-60 days after planting.

Table 4-7. Definition of the magnetite treatments.

Treatment designation	Concentration of nanoparticles / µg ml^{-1}
C_1	0
C_2	20
C_3	40
C_4	60
C_5	80

4.4 Effect of coating *Bradyrhizobium japonicum* with magnetite nanoparticles on total dry matter (vegetative components of soybean plant

The treatments with nanoparticles are given in Table 4-6. The magnetite nanoparticles were added to the liquid medium before autoclaving. Before stationary phase seeds were inoculated with 1-2 µl $seed^{-1}$ of liquid medium containing 10^6 or 10^7 *B. japonicum* ml^{-1} liquid medium based on treatments. The statistical design was randomized block design (RBD) with four blocks. The variables measured were dry weight leaf, stem, root, nodule, number of nodules, nodes, number of branches and plant height, measuring time was 70-90 days after planting (before flowering phase).

4.5 Acetylene reduction as an indicator of nitrogen fixation

4.5.1 Effect of magnetite nanoparticles on quantity of acetylene reduction (indicator of fixed nitrogen)

The treatments and conditions were as in section 4.4 above. The variable measured was quantity of C_2H_4 reduced, 60-70 days after planting.

4.5.2 Effect of nanobiocomposites on quantity of acetylene reduction (indicator of fixed nitrogen)

The treatments with nanoparticles are given in Table 4-8. The set up was the same as in section 4.4. The variable measured was quantity of C_2H_4 reduced, 60-70 days after planting.

Table 4-8. Definition of the magnetite treatments.

Treatment designation
T_1= B†. without nanobiocomposite
T_2= B. + pure magnetite nanoparticle
T_3= B. + magnetite soylecithin nanobiocomposite
T_4= B. + magnetite methylcellulose nanobiocomposite
T_5= B. + magnetite PVA‡ nanocomposite

† *Bradyrhizobium japonicum,* ‡ polyvinyl alcohol.

5 Results

5.1 Fourier transform infrared (FTIR) spectra analysis

Figure 5-1.shows the FTIR spectra of pure magnetite nanoparticles (Fe_3O_4), modified Fe_3O_4 with soylecithin, methylcellulose and poly-vinyl-alcohol (PVA). The characteristic absorption peak for Fe_3O_4 is observed at around 580 cm^{-1} and this results is consistant with previous studies [22], and those of the polymers are evident at 1060 cm^{-1} (C—O—C), 1704 cm^{-1}(C═O), 857 cm^{-1} (C═C—H), 1621 cm^{-1}, 1636 cm^{-1} and 1413 cm^{-1}(C═C), 2950 cm^{-1}(═C—H), ≈3400 cm^{-1} (N—H) [57]. These results verify that the Fe_3O_4 nanoparticles are coated with these polymers.

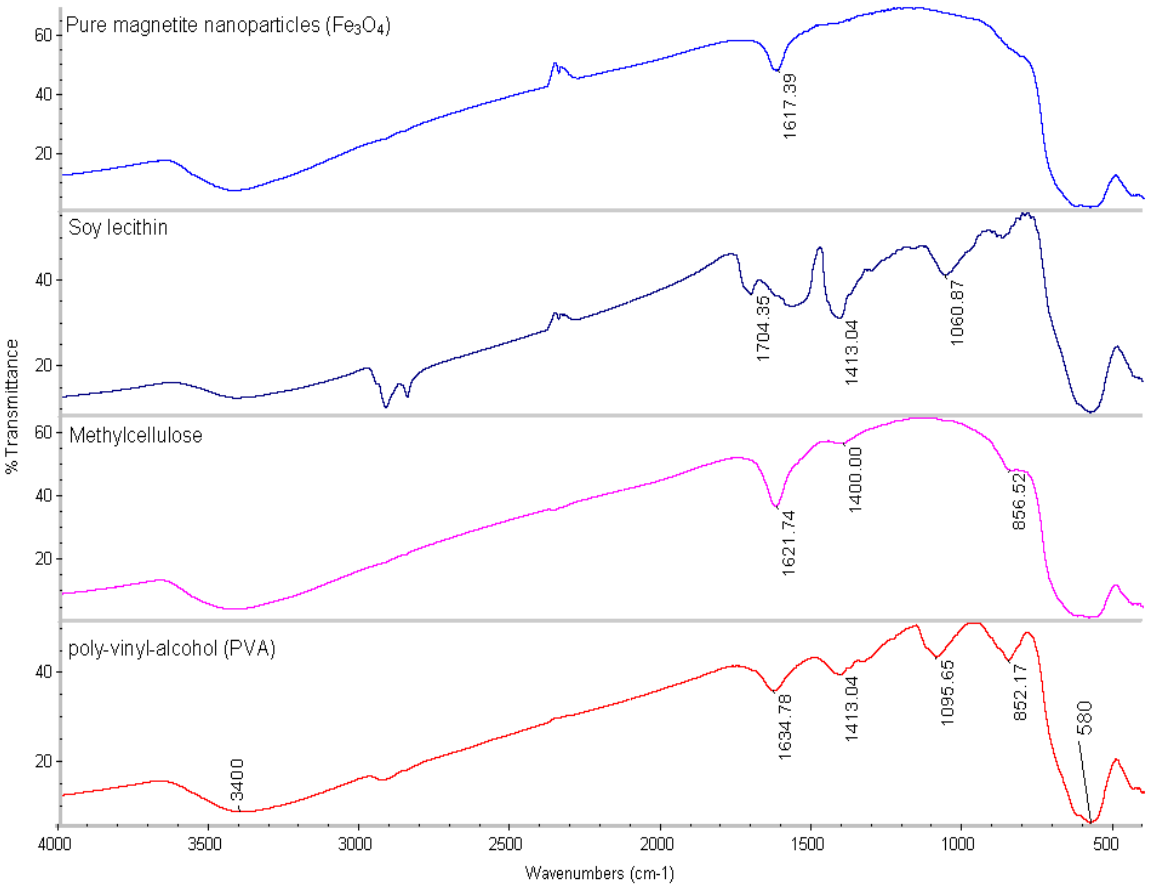


Figure 5-1. FTIR spectra of pure Fe_3O_4, soylecithin, methylcellulose, and PVA.

5.2 X-ray diffraction (XRD)

In order to determinte the crystalline nature of iron oxide nanoparticles and the nanocomposite, XRD analysis was performed. The results are shown in the Figure 5-2. The characteristic peaks generated within the magnetite can clearly be seen. No other crystalline peaks are apparent though the polymer component may be contributing to the amorphous halo between 15 and 30˚. The broadening of XRD peaks of pure nanoparticles appeared at 2θ: 30.50, 35.80, 43.50, 57.50, and 63.00. The peaks of nanobiocomposites did not shift but they were broader and higher, and also from comparing them is evident that the crystallographic structure of magnetite nanoparticles was unchanged after coating with polymers. The broadening of XRD peaks is therefore predominantly attributed to the size of nanoparticles, crystalline and/or amorphous, and source of polymer, which are directly related to the change in particles properties. Full-width half-maxima were measured on the magnetite peaks on each graph and following subtraction of the contribution from instrumental broadening, the corresponding crystallite size was calculated using the Scherrer equation. The sizes thus calculated are shown in Table 5-1, and these results are consistent with previous studies [10,139, 263].

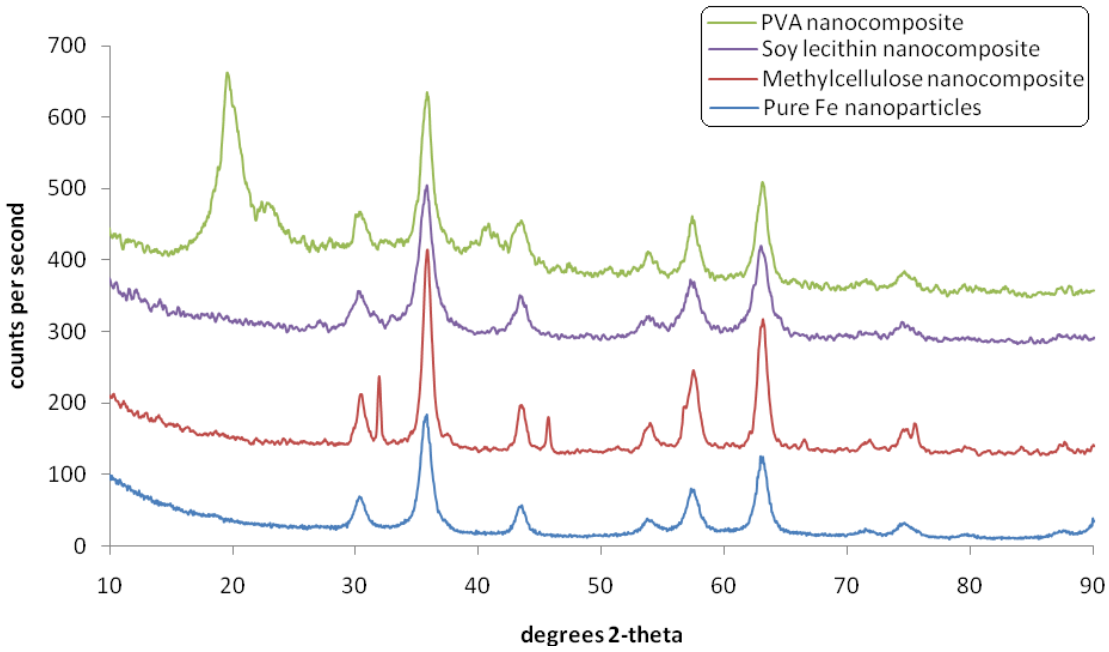


Figure 5-2. X-ray diffractogram of pure Fe_3O_4, soylecithin, methylcellulose, and PVA.

Table 5-1. Calculated crystallite size for pure magnetite nanoparticles and each polymer/magnetite nanobiocomposite.

Sample	Crystallite size / nm
Pure magnetite nanoparticles	8.2
Soylecithin/magnetite nanobiocomposite	6.1
Methylcellulose/magnetite nanobiocomposite	12.1
PVA/magnetite nanocomposite	7.4

5.3 Effects of magnetite nanoparticles on *Bradyrhizobium* growth rate

TEM of magnetite nanoparticles mixed with *Bradyrhizobium* (Figure 5-3) shows that nanoparticles are associated with the surface of *Bradyrhizobium*.

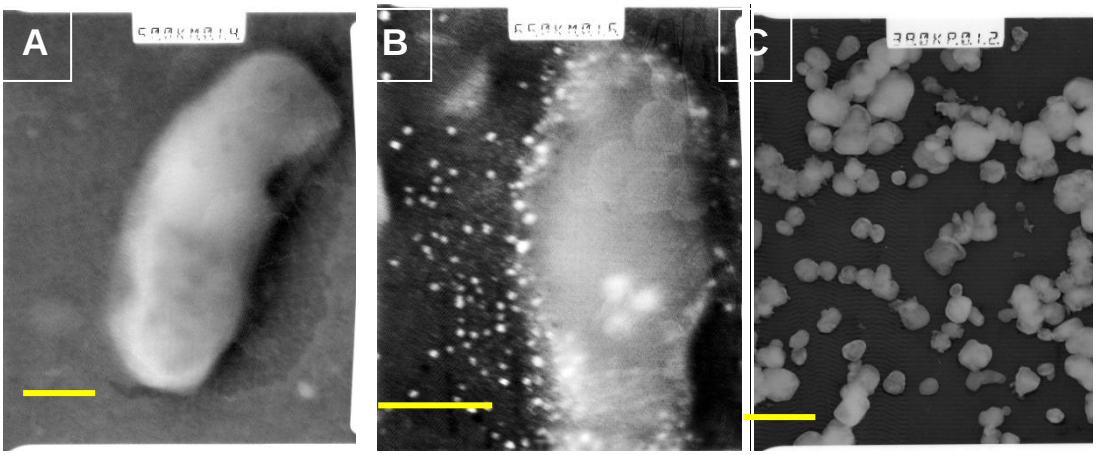
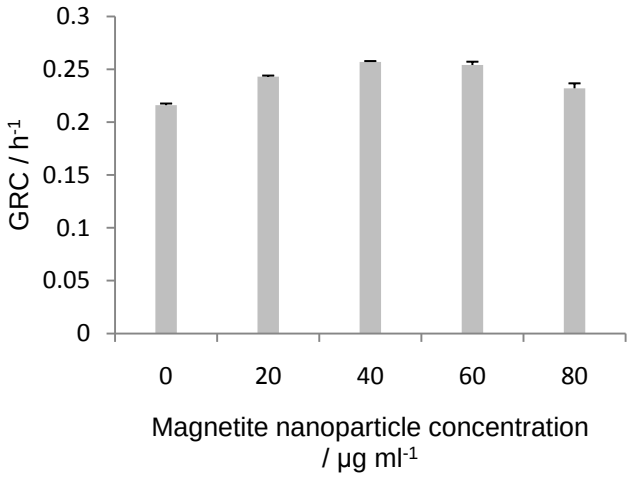


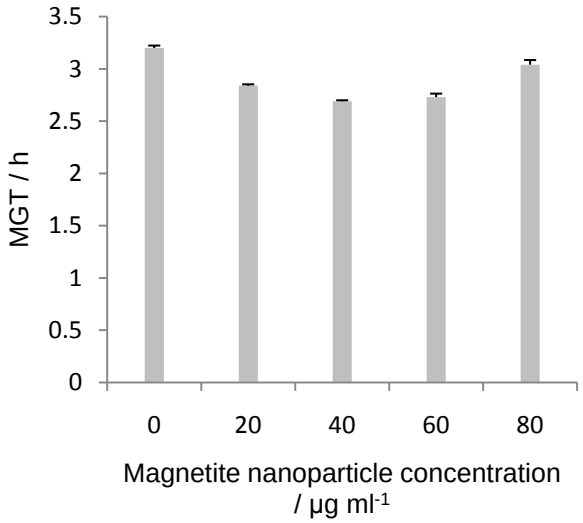
Figure 5-3. Transmission electron micrographs of: (A) *Bradyrhizobium japonicum* (scale bar, 350 nm); (B) *Bradyrhizobium japonicum* mixed with magnetite nanoparticles (scale bar, 435 nm); (C) magnetite nanoparticles, (scale bar, 29 nm).

Table 5-2 shows maximum growth of *Bradyrhizobium* cells at the different nanoparticle concentrations, and Figure 5-4. summarizes the growth indices.

Table 5-2. Effect of magnetite nanoparticle concentration on maximum growth of *B. japonicum* Histic.

Magnetite nanoparticle concentration / μg ml^{-1}	Maximum number of viable cells	MGT / h	GRC / h^{-1}	NG
Control (0)	6×10^8 (after 120 h)	3.2	0.21	17
20	7×10^8 (after 120 h)	2.8	0.24	21
40	10^9 (after 72 h)	2.6	0.26	22
60	10^9 (after 144 h)	2.6	0.25	22
80	10^8 (after 144 h)	3.1	0.22	19





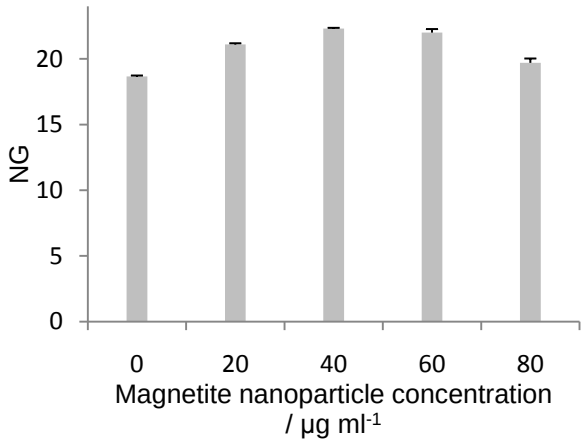


Figure 5-4. Effect of nanoparticle concentration on (top) the growth rate constant (GRC), (centre) mean generation time (MGT), and (bottom) number of generations (NG) of *Bradyrhizobium japonicum*. Error bars show standard error.

40 of magnetite nanoparticles enabled the maximum number of viable cells per ml to be reached at 72 h, while the other treatments required about 2-3 days (Table 5-2). However, the results illustrated that treatments 60 and 80 µg ml^{-1} at 144 h gave the maximum N_V (10^9 and 10^8 viable cells ml^{-1}, respectively, see Table 5-2).

According to the analysis of variation (Table 5-3), magnetite nanoparticles in the liquid media significantly increased the growth rate constant and the number of generations, and decreased the mean generation time (Figure 5-4).

Table 5-3. Mean squares from the analysis of variation of the growth indices of *Bradyrhizobium japonicum* Histic in the log phase of growth.

Source of Variation	df	Mean squares		
		MGT/ h	GRC / h^{-1}	NG
Blocks	3	0.003	0.00004	0.165
Treatments	4	0.190**	0.0011**	9.561**
Residual	12	0.003	0.00003	0.161

** *F* test indicated significance at $P < 0.01$.

However, comparisons of the treatments' means showed that 40 and 60 µg ml^{-1} of the nanoparticles had a positive effect on mean of the mean generation time (MGT), number of generations (NG), and growth rate constant (GRC), in addition effects of the nanoparticles (20, 40, and 60 µg ml^{-1}) on the MGT and effects of the oncentrations (40, and 60 µg ml^{-1}) on the GRC and the NG were similar at alpha = 0.01 (Tables 5-4, 5, and 6). In addition, the comparing of treatment means during the growth phases showed that the growth indices (MGT, NG and GRC) subject to the control treatment (without nanoparticle) were less than other treatments (Tables 5-4, 5, and 6).

Table 5-4. Comparison of the MGT means of *Bradyrhizobium japonicum* Histic under magnetite nanoparticle concentration effect.

Magnetite nanoparticle concentration / µg ml^{-1}	Mean of mean generation time (MGT) / h	LSD value =0.08439	LSD value =0.1183
		Alpha = 0.05	Alpha =0.01
Control (0)	3.201	A	a
20	2.844	C	c
40	2.689	D	d
60	2.727	D	cd
80	3.037	B	b

Table 5-5. Comparison of the GRC means of *Bradyrhizobium japonicum* Histic under magnetite nanoparticle concentration effect.

Magnetite nanoparticle concentration / µg ml^{-1}	Mean of growth rate constant (GRC) / h^{-1}	LSD value =0.002668	LSD value =0.003741
		Alpha = 0.05	Alpha =0.01
Control (0)	0.2166	E	d
20	0.2437	C	b
40	0.2576	A	a
60	0.2543	B	a
80	0.2329	D	c

Table 5-6. Comparison of the NG means of *Bradyrhizobium japonicum* Histic under magnetite nanoparticle concentration effect.

Magnetite nanoparticle concentration / µg ml^{-1}	Mean of number of generations (NG)	LSD value =0.6182	LSD value =0.8666
		Alpha = 0.05	Alpha =0.01
Control (0)	18.66	D	d
20	21.10	B	b
40	22.29	A	a
60	22.01	A	a
80	19.69	C	c

Measurement of pH in the growth stages of *Bradyrhizobium* showed that all treatments (except the control treatment) could maintain the pH near the optimum (pH=6.8±0.2) for bacteria growth (Table 5-7). This suggests that the magnetite nanoparticles can complex OH^- ions in the medium as they accumulate from

bacterial secretions, and also the nanoparticle compensate iron deficiency when raise pH of medium [81,119,194].

Table 5-7. Variation of pH† during growth of *B. japonicum* in medium in presence of magnetite nanoparticle.

Magnetite nanoparticle concentrations / μg ml^{-1}	Growth times / h							
	0	24	48	72	96	120	144	168
Control (0)	6.80	7.00	7.50	7.20	7.10	6.90	6.90	7.70
20	6.80	6.80	6.90	7.10	6.60	6.50	6.40	6.40
40	6.80	6.80	6.86	6.78	6.70	6.50	6.45	6.43
60	6.80	6.80	6.82	6.73	6.62	6.45	6.34	6.27
80	6.80	6.80	6.77	6.70	6.55	6.45	6.30	6.21

† Uncertainly of pH determination is ± 0.02.

5.3.1 Discussion

The main question to answer is how the presence of the magnetite nanoparticles in the medium affects the growth. The following possible explanations seem worth considering:

1. The pH results (Table 5-7) suggest that the nanoparticles have a pH buffering effect, which could be achieved either by adsorbing alkaline bacterial secretions, or by reacting with them.

2. The literature suggests that nanoparticles catalyse reactions tending to keep the pH neutral; e.g., K_2HPO_4 complexing with $HC_2H_3O_2$ [81, 91,93,119,140,176,194].

3. The literature suggests that nanoparticles may complex and inactivate oxygen scavengers in the medium, hence increasing oxygen availability [81,140,176,194].

4. Increasing concentration of the nanoparticles could be induced releasing of ferric uptake repressor (Fur) proteins of the bacterial cells as inhibitor of the nanoparticle effect [27].

5.3.2 Conclusions

Bradyrhizobium growing indices are affected by magnetite nanoparticles. Although increasing concentrations of nanoparticles from 20 to 60 µg ml^{-1} had a favourable effect on the *Bradyrhizobium* growing indices, a concentration of 80 µg ml^{-1} was unfavourable (MGT increased and NG and GRC diminished). The magnetite nanoparticles had the effect of increasing the number of harvestable bacterial cells, and bacterial growth vigour, and promoted survival via protection of the conditions of multiplication.

5.4 Effects of pH and magnetite nanoparticles on *Bradyrhizobium* growth rate

5.4.1 Population of viable bacterial cells

According to the analysis of variation (Table 5-8) the population of the viable cells was affected both nanoparticles and pH levels. Main effects of magnetite nanoparticle concentration and the pH significantly increase the bacterial population. However, interaction effect between the nanoparticles and the pH levels was not significance (Table 5-8).

Table 5-8. Mean square from analysis of variation of log number of viable cell of the *Bradyrhizobium japonicum* Histic.

Source of Variation	df	Mean squares of log number of viable cells (N_v)
Blocks	7	85
Nanoparticles (N_P)	2	11**
pH	5	3.1**
N_P × pH	10	$0.3^{n.s.}$
Residual	119	0.2

** *F* test indicated significance at $P < 0.01$; and $^{n.s.}$ indicates a nonsignificant result.

Maximum number of viable cells was obtained by 40 µg ml^{-1} of the nanoparticles at 120 h about 10^8 viable cells ml^{-1}(Table 5-9) (Figure 5-5). However, the lowest number of viable cells was obtained by 80 µg ml^{-1} of the nanoparticles (Table 5-9). Measured the log number of viable cells by the control treatment and 80 µg ml^{-1} of the nanoparticles showed that the bacterial growth had a temperary cessation for one day (from 24 to 48 h) (Figure 5-5).

Table 5-9. Effect of magnetite nanoparticle concentration on maximum number of viable cells at 120 h of *Bradyrhizobium japonicum* Histic.

Magnetite nanoparticle concentration / µg ml^{-1}	Maximum number of viable cells at 120 h
Control (0)	5×10^7
40	10^8
80	10^7

Clearly maximum population of the bacteria was occurred at pH=6 and 7. The number of viable cells at pH=8 was higher than pH=4 and 9 (Table 5-10).

Table 5-10. Effect of pH levels on the maximum number of viable cells at 120 h of *Bradyrhizobium japonicum* Histic.

pH	Maximum number of viable cells at 120 h
4	2×10^7
5	4×10^7
6	10^8
7	5×10^7
8	3×10^7
9	2×10^7

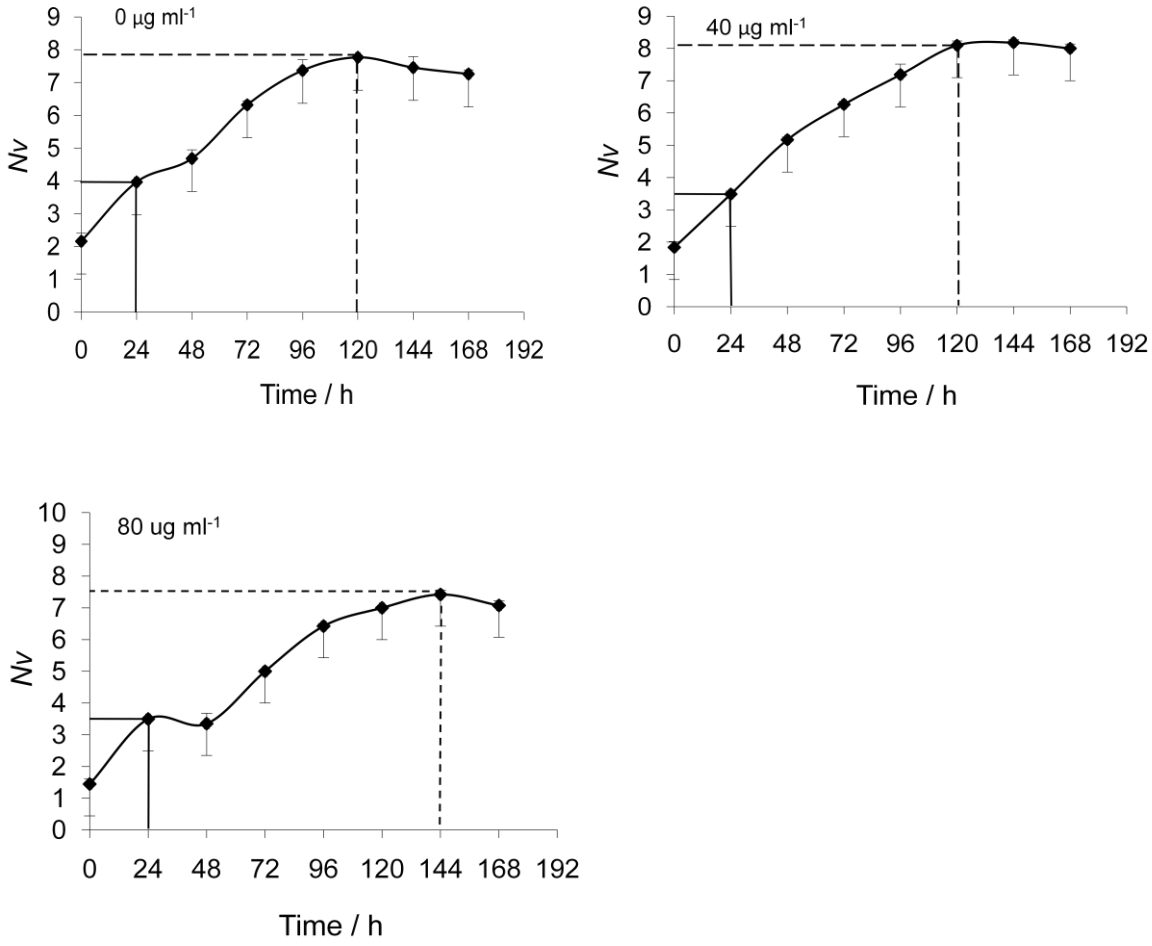


Figure 5-5. Variation of log number of viable cells per ml^{-1} (N_v) at the different concentrations of magnetite nanoparticles (average over all pH levels). Error bars show standard error.

Effects of the pH levels on the number of viable cells showed that at initial time of growth, pH= 9 and 4 had minimum of the number of viable cells (Figure 5-6). The results showed that duration of stationary phase was long from the pH = 5 to 9 and they could preserve the survival for about 4 days, while this duration for pH=4 was about 3 days (Figure 5-6). The maximum number of viable cells was obtained by pH=6 after 96 h. However, at the pH=4 and 9, the growth of bacterial cells during interval times, showed that their growth had temporary cessations compared to the other pH levels (Figure 5-6).

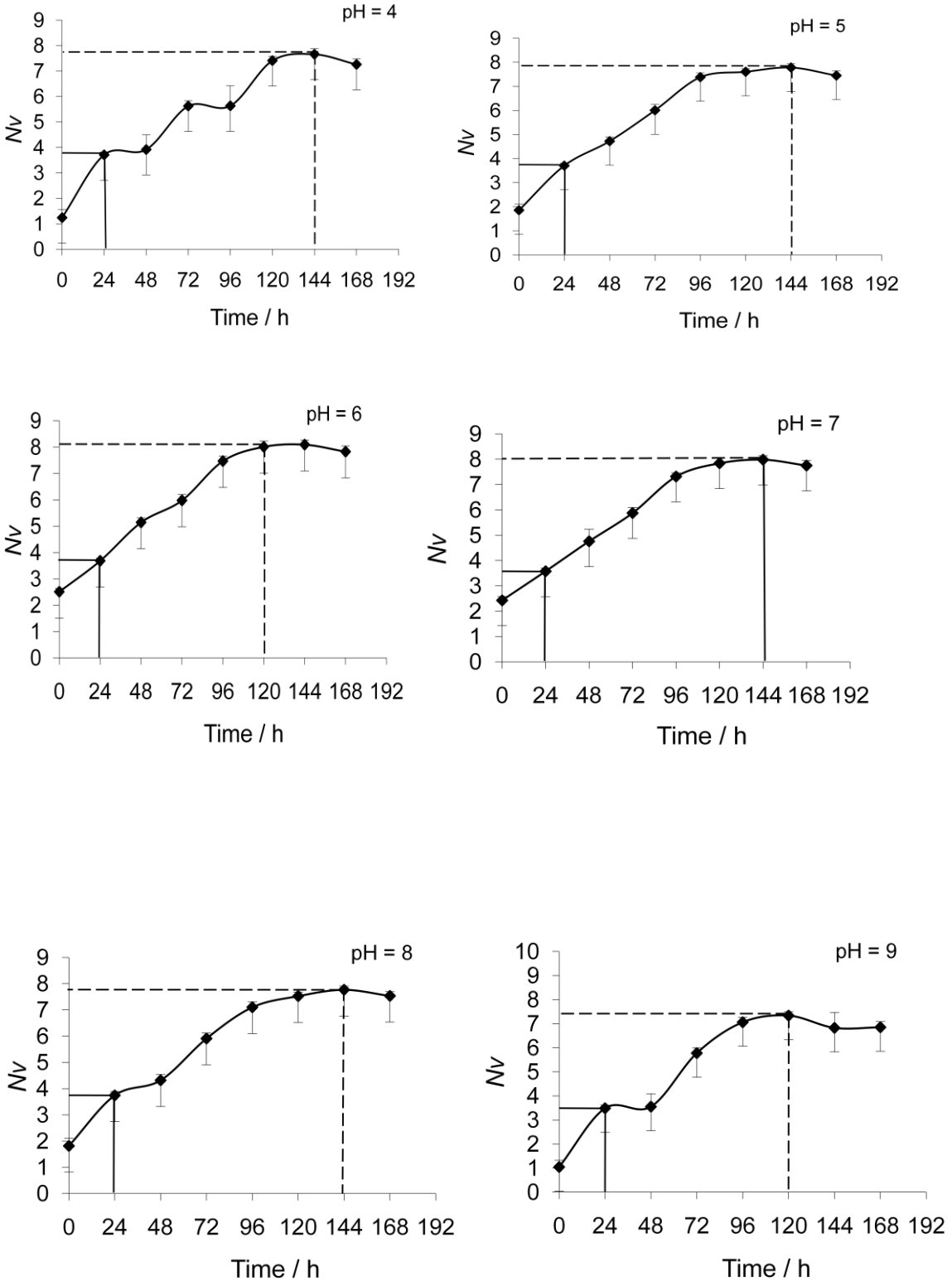


Figure 5-6. Variation of log number of viable *B. japonicum* cells per ml (N_v) at different pHs (average over all nanoparticle concentrations). Error bars show standard error.

5.4.2 Mean generation time (MGT)

Analysis of variation (Table 5-11) showed that growth indices (MGT, GRC and NG) of *Bradyrhizobium japonicum* Histic were affected both magnetite nanoparticle concentration and pH, and also the growth indices responded to interaction effect between the nanoparticles and pH (Table 5-11).

Table 5-11. Mean squares from analysis of variation of the physiological indices growth of the coated *Bradyrhizobium japonicum* Histic.

Source of Variation	df	Mean squares		
		MGT/ h	GRC h^{-1}	NG
Blocks	3	0.16	0.00002	0.45
Nanoparticles (N_P)	2	23**	0.00491**	94.1**
pH	5	1.5**	0.00044**	8.5**
N_P × pH	10	1.3**	0.00039**	7.5**
Residual	51	0.333	0.00005	0.95

** *F* test indicated significance at $P < 0.01$, and $^{n.s.}$ indicates a nonsignificant result.

40 µg ml^{-1} of the nanoparticles had a more positive effect on the MGT than other treatments (Figure 5-7). The results showed that the lowest mean of MGT, was occurred at pH=6 and 7. Likewise, the lowest mean of MGT was obtained by interaction effects between 40 µg ml^{-1} of the nanoparticles and pH=6 and 7(Figure 5-7).

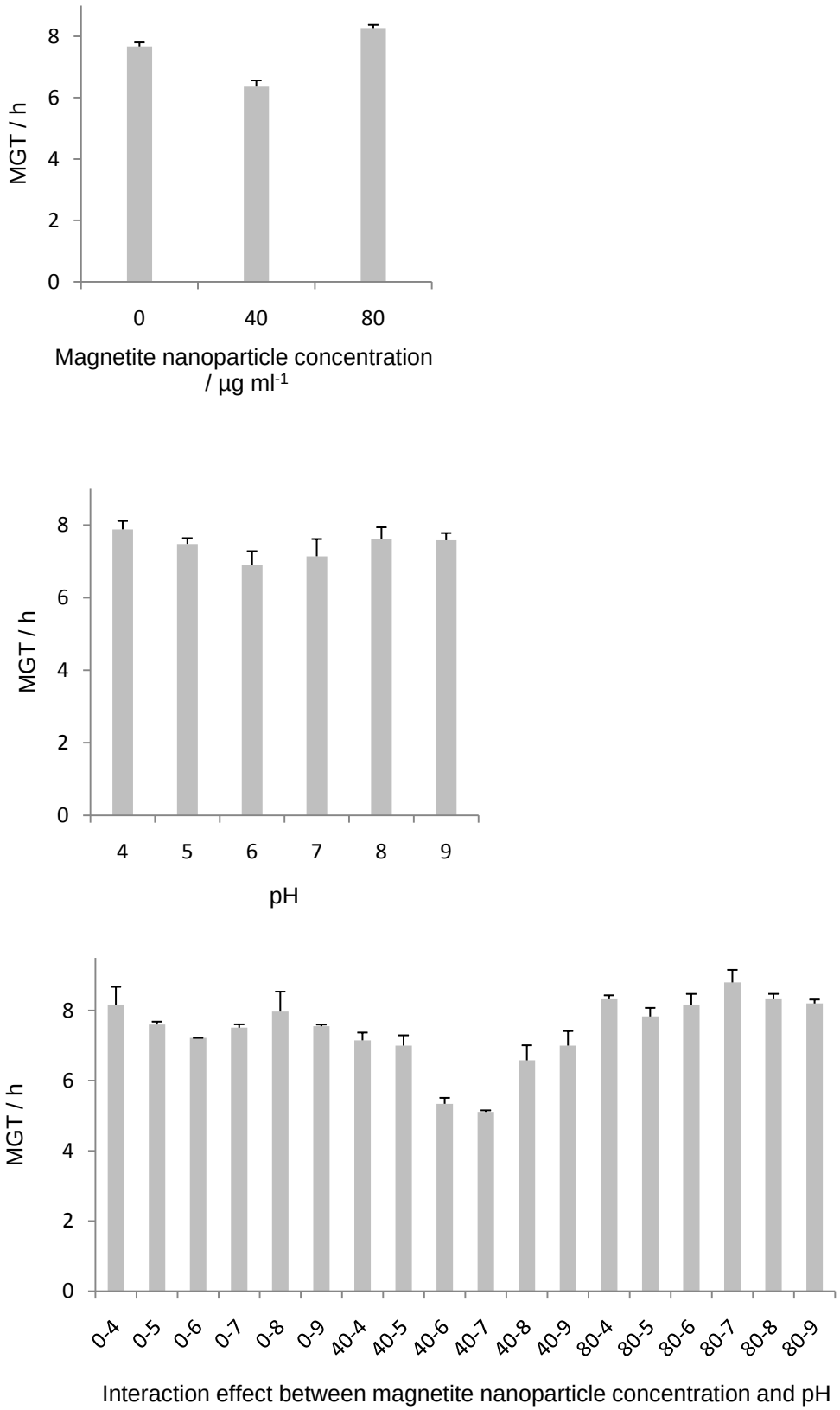


Figure 5-7. Effect of (top) magnetite nanoparticle concentration (average over all pH levels), (centre) pH (average over all nanoparticle concentrations), and (bottom) their interaction (labelled as $N_{P\ conc.}$-pH) on the mean generation time (MGT) of *Bradyrhizobium japonicum* Histic, error bars show standard error.

5.4.3 Growth rate constant (GRC)

The GRC was affected by nanoparticles, pH and their interaction effects (Table 5-11). The greatest of GRC was obtained by 40 µg ml^{-1} of nanoparticles, pH= (6 and 7) and interaction effect between 40 µg ml^{-1} of nanoparticles and pH= (6 and 7) (Figure 5-8).

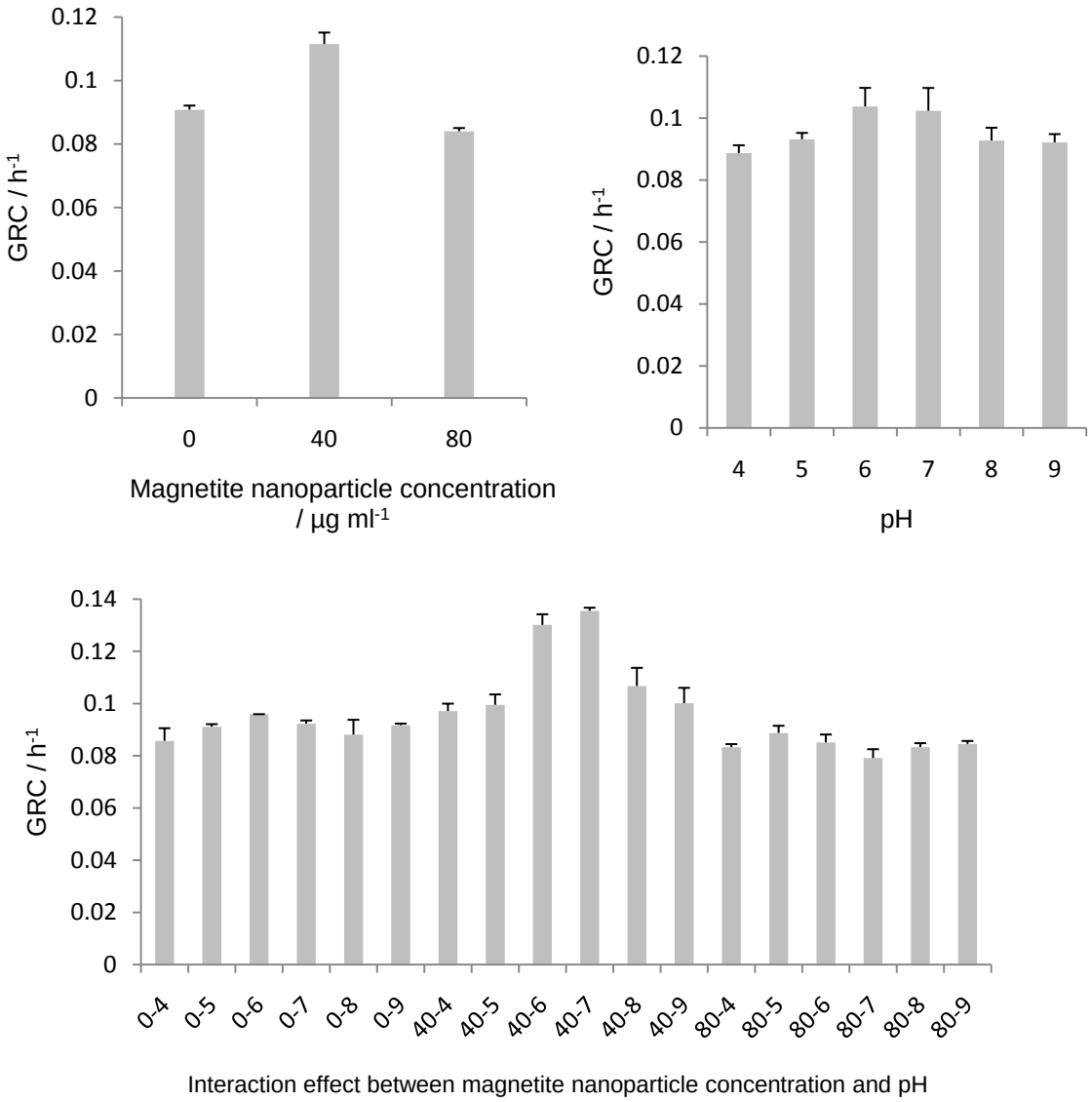


Figure 5-8. Effect of (left upper) magnetite nanoparticle concentration (average over all pH levels), (right upper) pH (average over all nanoparticle concentrations), and (bottom) their interaction (labelled as $N_{P\ conc.}$-pH) on the growth rate constant (GRC) of the *Bradyrhizobium japonicum* Histic. Error bars show standard error.

5.4.4 Number of generations (NG)

Effects of nanoparticles, pH and their interaction effects on the NG of *Bradyrhizobium japonicum* Histic were significant, at $P<0.01$ in the liquid media (Table 5-11). Figure 5-9 shows the greatest NG occurred with 40 µg ml^{-1} of nanoparticles, pH= (6 and 7) and maintained in the interaction effect between 40 µg ml^{-1} of nanoparticles and pH= (6 and 7) (Figure 5-9).

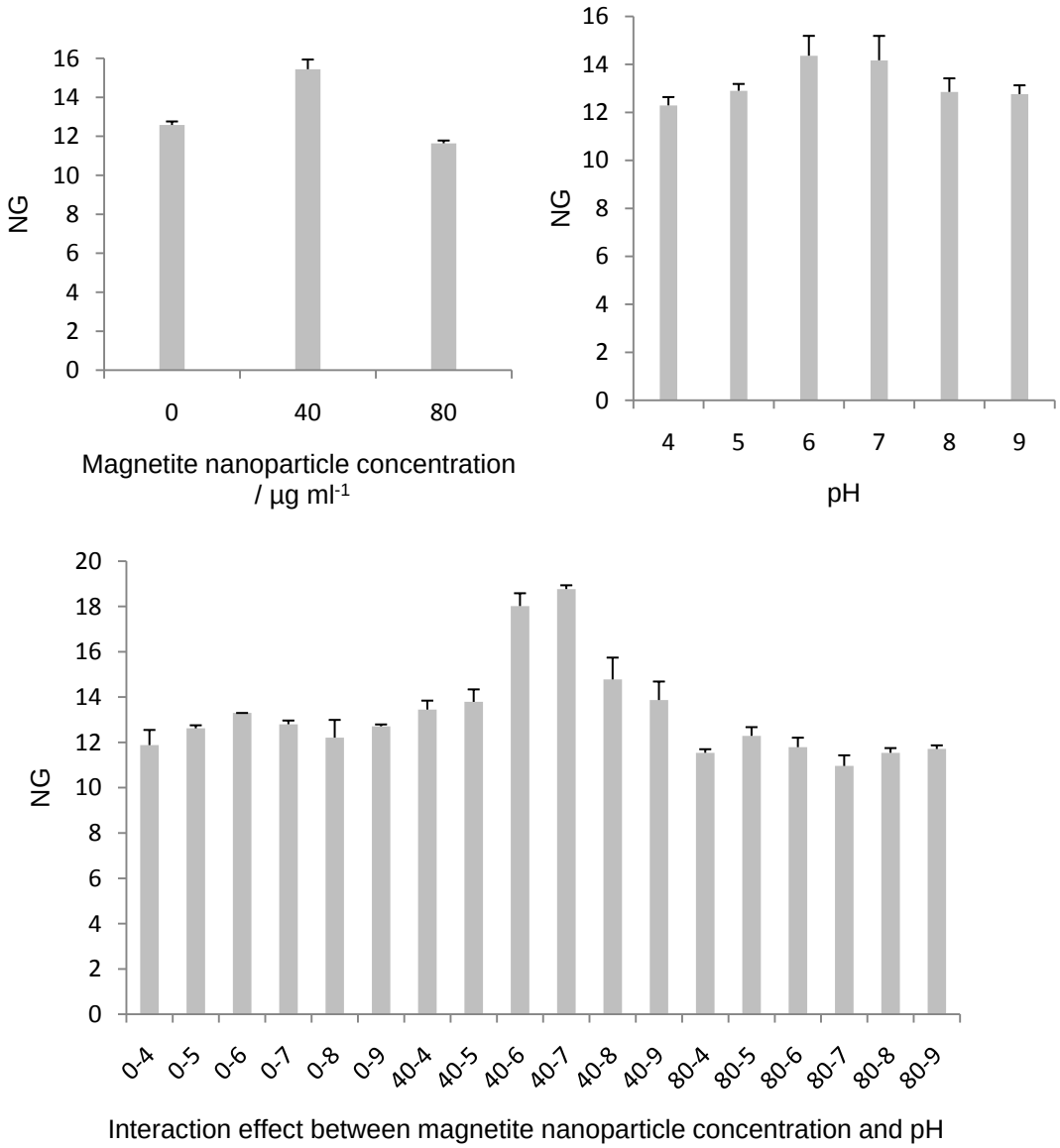


Figure 5-9. Effect of (left upper) magnetite nanoparticle concentration (average over all pH levels), (right upper) pH (average over all nanoparticle concentrations), and (bottom) their interaction (labelled as $N_{P\ conc.}$-pH) on the number of generations (NG) of *Bradyrhizobium japonicum* Histic. Error bars show standard error.

5.4.5 Comparison of the growth indices means of *Bradyrhizobium japonicum* Histic under magnetite nanoparticle, pH and their interaction effects

The growth indices (MGT, GRC and NG) means under magnetite nanoparticle effect were compared based on least significant differences (LSD), and the results showed that the differences of treatments means were significant, and each treatment had an individual rank (Tables 5-12,13 and 14).

Table 5-12. Comparison of the MGT means of *Bradyrhizobium japonicum* Histic under magnetite nanoparticle concentration effect.

Magnetite nanoparticle concentration / µg ml^{-1}	Mean of mean generation time (MGT) / h	LSD value =0.3344	LSD value =0.0.4457
		Alpha = 0.05	Alpha =0.01
0	7.673	B	b
40	6.364	C	c
80	8.275	A	a

Table 5-13. Comparison of the GRC means of *Bradyrhizobium japonicum* Histic under magnetite nanoparticle concentration effect.

Magnetite nanoparticle concentration / µg ml^{-1}	Mean of growth rate constant (GRC) / h^{-1}	LSD value =0.004082	LSD value =0.005440
		Alpha = 0.05	Alpha =0.01
0	0.091	B	b
40	0.112	A	a
80	0.084	C	c

Table 5-14. Comparison of the NG means of *Bradyrhizobium japonicum* Histic under magnetite nanoparticle concentration effect.

Magnetite nanoparticle concentration / µg ml^{-1}	Mean of number of generations (NG)	LSD value =0.5655	LSD value =0.7536
		Alpha = 0.05	Alpha =0.01
0	12.58	B	b
40	15.45	A	a
80	11.64	C	c

Comparisons of the mean of MGT showed that the greatest differences of the average of MGT was between pH= 6 and pH=4 (Tables 5-15). Comparisons of the GRC means and the NG means under pH levels showed that pairs pH levels ((4 and 5), (6 and 7) and (8 and 9)) were the same as, while each pair of the treatments was independent to other treatments (Tables 5-16 and 17).

Table 5-15. Comparison of the MGT means of *Bradyrhizobium japonicum* Histic under pH effect.

pH	Mean of mean generation time (MGT) / h	LSD value =0.4730	LSD value =0.6304
		Alpha =0.05	Alpha =0.01
4	7.881	A	a
5	7.479	AB	abc
6	6.913	C	c
7	7.147	BC	bc
8	7.624	A	ab
9	7.584	AB	ab

Table 5-16. Comparison of the GRC means of *Bradyrhizobium japonicum* Histic under pH effect.

pH	Mean of growth rate constant (GRC) / h^{-1}	LSD value =0.0.005772	LSD value =0.007693
		Alpha =0.05	Alpha =0.01
4	0.089	B	b
5	0.093	B	b
6	0.104	A	a
7	0.102	A	a
8	0.093	B	b
9	0.092	B	b

Table 5-17. Comparison of the NG means of *Bradyrhizobium japonicum* Histic under pH effect.

pH	Mean of number of generations (NG)	LSD value =0.7997	LSD value =1.066
		Alpha =0.05	Alpha =0.01
4	12.29	B	b
5	12.90	B	b
6	14.37	A	a
7	14.17	A	a
8	12.85	B	b
9	12.76	B	b

Comparisons of the growth indices (MGT, GRC and NG) means under interaction effect between nanoparticles and pH showed that the lowest MGT and the greatest NG and GRC means obtained by interaction effects between 40 µg ml^{-1} of the nanoparticles with pH=6 and 7 compared to the other interactions (Table 5-18, 19 and 20). However, the comparisons of interaction effects showed that the nanoparticles effect in the combination of treatments was more considerable than pH effect on the growth indices (Table 5.18, 19 and 20).

Table 5-18. Comparison of the MGT means of *Bradyrhizobium japonicum* Histic under interaction effect between magnetite nanoparticle concentration and pH.

Interaction effect between magnetite nanoparticle concentration and pH	Mean of mean generation time (MGT) / h	LSD value =0.8192	LSD value =1.092
		Alpha = 0.05	Alpha = 0.01
0-4	8.168	AB	abc
0-5	7.607	BCD	bcde
0-6	7.224	CDE	cde
0-7	7.509	BCD	bcde
0-8	7.972	BC	abcd
0-9	7.555	BCD	bcde
40-4	7.154	CDE	cde
40-5	6.998	DE	de
40-6	5.343	F	f
40-7	5.116	F	f
40-8	6.577	E	e
40-9	6.994	DE	de
80-4	8.320	AB	ab
80-5	7.833	BC	abcd
80-6	8.172	AB	abc
80-7	8.802	A	a
80-8	8.324	AB	ab
80-9	8.202	AB	abc

Table 5-19. Comparison of the GRC means of *Bradyrhizobium japonicum* Histic under interaction effect between magnetite nanoparticle concentration and pH.

Interaction effect between magnetite nanoparticle concentration and pH	Mean of growth rate constant (GRC) / h^{-1}	LSD value =0.009998	LSD value =0.01332
		Alpha = 0.05	Alpha = 0.01
0-4	0.086	EF	def
0-5	0.091	CDE	cdef
0-6	0.096	CD	bcde
0-7	0.092	CDE	cdef
0-8	0.088	DEF	cdef
0-9	0.092	CDE	cdef
40-4	0.097	BCD	bcd
40-5	0.010	BC	bc
40-6	0.130	A	a
40-7	0.136	A	a
40-8	0.110	B	b
40-9	0.100	BC	bc
80-4	0.083	EF	ef
80-5	0.089	DEF	cdef
80-6	0.085	EF	def
80-7	0.079	F	f
80-8	0.083	EF	ef
80-9	0.085	EF	def

Table 5-20. Comparison of the NG means of *Bradyrhizobium japonicum* Histic under interaction effect between magnetite nanoparticle concentration and pH..

Interaction effect between magnetite nanoparticle concentration and pH	Mean of number of generations (NG)	LSD value =1.385	LSD value =1.846
		Alpha = 0.05	Alpha = 0.01
0-4	11.88	EF	def
0-5	12.62	CDE	cdef
0-6	13.29	CD	bcde
0-7	12.79	CDE	cdef
0-8	12.21	DEF	cdef
0-9	12.71	CDE	cdef
40-4	13.46	BCD	bcd
40-5	13.79	BC	bc
40-6	18.02	A	a
40-7	18.77	A	a
40-8	14.78	B	b
40-9	13.87	BC	bc
80-4	11.54	EF	ef
80-5	12.29	DEF	cdef
80-6	11.79	EF	def
80-7	10.96	F	f
80-8	11.54	EF	ef
80-9	11.71	EF	def

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5.4.6 Response of water activity (a_w) to magnetite nanoparticle concentration and pH

According to the analysis of variation (Table 5-21) the a_w was affected by main effects of nanoparticle concentrations and the pH levels, while their interaction had no significant effect on the a_w. Magnetite nanoparticles increased the a_w than the control (Figure 5-10). Moreover, the a_w had increased after 7 days, and it was greater in the neutral and alkaline pH than control. However maximum variation of the a_w occurred at pH than 8 and 9 during 7 days (Figure 5-10).

Table 5-21. Mean squares from analysis of variation of the water activity (a_w).

Source of Variation	df	Mean squares	
		Zero time	After 7 days
Blocks	3	0.000012	0.000004
Nanoparticles (N_P)	2	0.000014**	0.000011*
pH	5	0.000024**	0.000010*
N_P × pH	10	$0.000003^{n.s.}$	$0.000004^{n.s.}$
Residual	51	0.000004	0.000002

*, ** *F* test indicated significance at $P< 0.05$ and $P< 0.01$, respectively;
$^{n.s.}$ indicates a nonsignificant result.

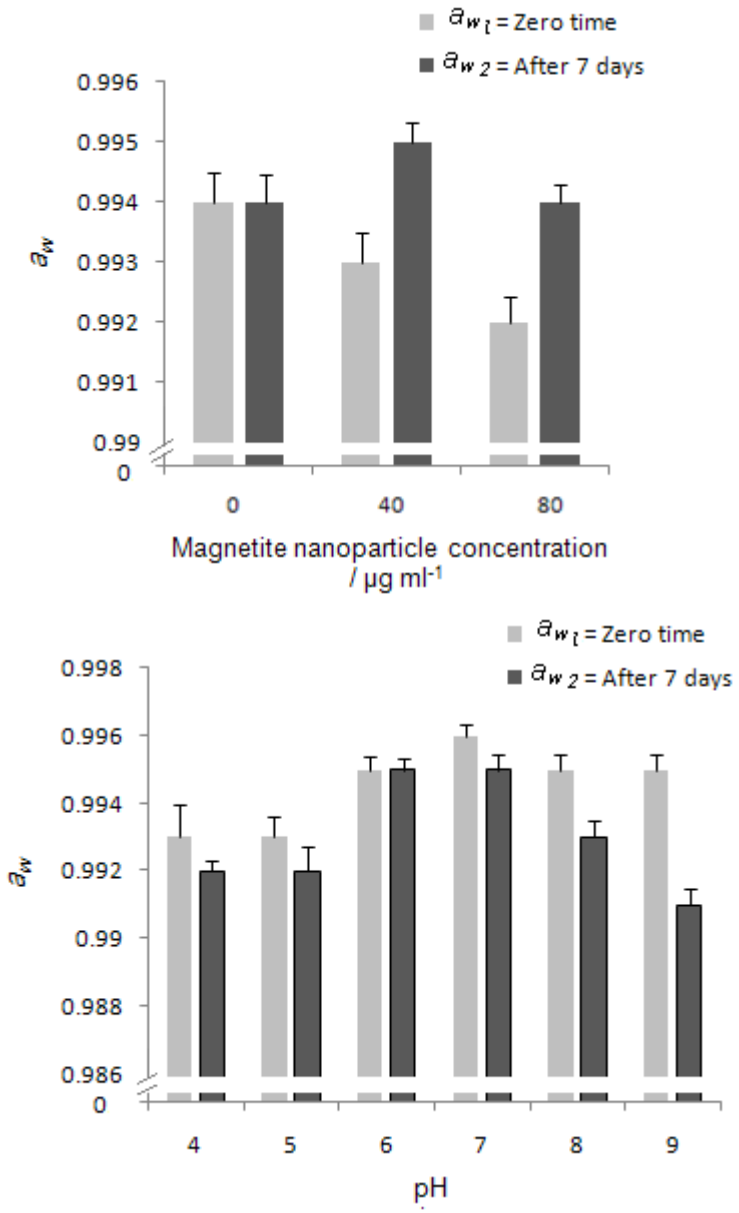

Figure 5-10. Response of water activity (a_w) to (top) the nanoparticles (average over all pH levels) and (bottom) the pH levels (average over all nanoparticle concentrations); gray bars and black bars indicate measured times at zero time and after 7 days of bacterial growth, respectively. Error bars show standard error.

5.4.6.1 Comparison of water activity (a_w) means under magnetite nanoparticle concentration and pH

Comparisons of water activity means under the nanoparticles effects showed that the greatest water activity obtained by the control treatment, however, the differences of water activity means between control treatment and 80 µg ml^{-1} of the nanoparticles, was significant at zero time (Table 5.22). In contrast, after 7 days, the greatest water activity was obtained by 40 µg ml^{-1} of the nanoparticles (Table 5-23).

Table 5-22. Comparison of water activity means at zero time under magnetite nanoparticle concentration effect in liquid culture media of *Bradyrhizobium japonicum* Histic.

Magnetite nanoparticle concentration / µg ml^{-1}	Mean of water activity (a_w) at zero time	LSD value =0.0011	LSD value =0.0015
		Alpha = 0.05	Alpha =0.01
0	0.994	A	a
40	0.993	AB	ab
80	0.992	B	b

Table 5-23. Comparison of water activity means after 7 days under magnetite nanoparticle concentration effect in liquid culture media of *Bradyrhizobium japonicum* Histic.

Magnetite anoparticle concentration / µg ml^{-1}	Mean of water activity (a_w) after 7 days	LSD value =0.00089	LSD value =0.00118
		Alpha = 0.05	Alpha =0.01
0	0.994	B	ab
40	0.995	A	a
80	0.994	B	b

Comparisons of water activity means under pH effect showed that the greatest water activity was obtained at pH=6 and 7 compared to the other pHs investigated at zero time and after 7 days (Table 5-24 and 25). The lowest water activity at zero time was at pH=4 and 9, and after 7days, was occurred at pH=4 and 5, and also, the water activity decreased at different pH levels after 7 days (Table 5.24 and 25).

Table 5-24. Comparison of water activity means at zero time under pH effect in liquid culture media of *Bradyrhizobium japonicum* Histic

pH	Mean of water activity (a_w) At zero time	LSD value =0.0013 Alpha =0.05	LSD value =0.0017 Alpha =0.01
4	0.9931	C	b
5	0.9934	BC	b
6	0.9948	A	ab
7	0.9955	A	a
8	0.9946	AB	ab
9	0.9947	A	ab

Table 5-25. Comparison of water activity means after 7 days under pH effect in liquid culture media of *Bradyrhizobium japonicum* Histic.

pH	Mean of water activity (a_w) After 7 days	LSD value =0.00155 Alpha =0.05	LSD value =0.00206 Alpha =0.01
4	0.9920	BC	c
5	0.9928	BC	bc
6	0.9947	A	ab
7	0.9949	A	a
8	0.9930	B	abc
9	0.9914	C	c

5.4.7 Response of oxidation reaction potential (ORP) to magnetite nanoparticle concentration and pH

The ORP was affected by nanoparticles, pH, and their interaction effects at both measured times (zero time and after 7 days) (Table 5-26). In the control treatment, the ORP means increased after 7 days, whilst the effect of magnetite nanoparticles caused a decrease in the OPR after 7 days (Figure 5-11). The ORP means were decreased by the addition of the nanoparticles and by increasing pH, and the effect of 40 µg ml^{-1} of the nanoparticles was more than 80 µg ml^{-1} of the nanoparticles (Figure 5-11).

Table 5-26. Mean squares from analysis of variation of the oxidation reaction potential (ORP).

Sources of Variation	df	Mean squares	
		Zero time	After 7 days
Blocks	3	122	405
Nanoparticles (N_P)	2	1717**	234557**
pH	5	111588**	130344**
N_P × pH	10	437**	336**
Residual	51	94	123

*, ** F test indicated significance at $P< 0.05$ and $P < 0.01$, respectively.

The interaction between nanoparticle and pH was also significant (Table 5-26), and this was due to the reduction under high pH being significant larger wher when nanoparticles were present.

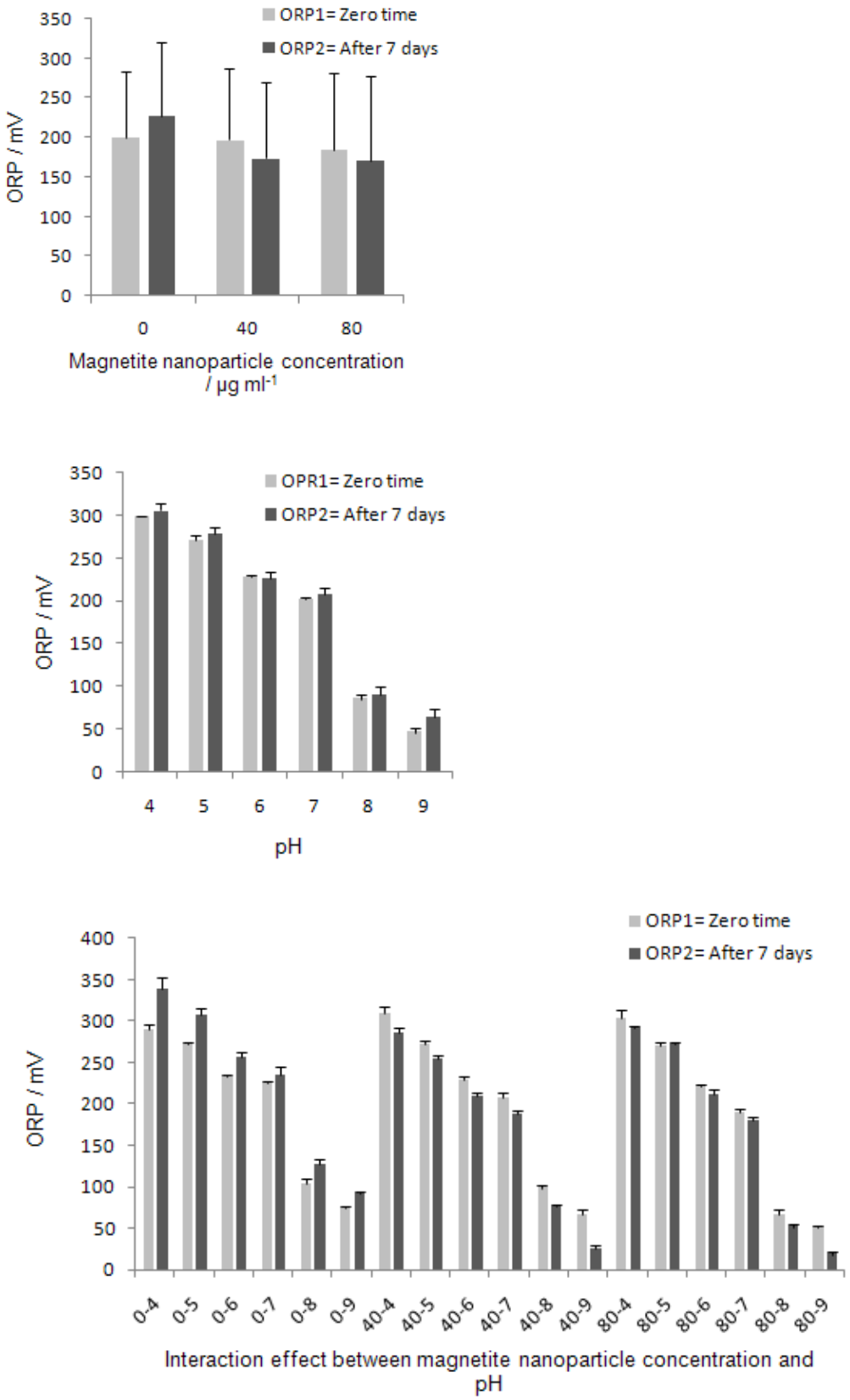

Figure 5-11. Response of the ORP to (top) the nanoparticles (average over all pH levels) and (centre) the pH levels (average over all nanoparticle concentrations) and (bottom) their interaction (labelled as $N_{P\ conc.}$-pH); gray bars and black bars indicate measured times at zero time and after 7 days of bacterial growth, respectively. Error bars show standard error.

5.4.7.1 Comparison of oxidation reaction potential (ORP) means under effects of magnetite nanoparticle concentration and pH

The results showed that at zero time, the difference of ORP means between the control treatment and 40 µg ml^{-1} of the nanoparticles was not significant, however, the lowest of ORP was obtained by 80 µg ml^{-1} of the nanoparticles at zero time (Table 5-27). After 7 days, the ORP means by the nanoparticles effects, was decreased, while by the control treatment increased (Table 5-28).

Table 5-27. Comparison of the ORP means at zero time under magnetite nanoparticle concentration effect in liquid culture media of *Bradyrhizobium japonicum* Histic.

Magnetite nanoparticle concentration / µg ml^{-1}	Mean of oxidation reaction potential (ORP) at zero time	LSD value =5.61 Alpha = 0.05	LSD value =7.57 Alpha =0.01
0	200	A	a
40	197	A	a
80	184	B	b

Table 5-28. Comparison of the ORP means after 7 days under magnetite nanoparticle concentration effect in liquid culture media of *Bradyrhizobium japonicum* Histic.

Magnetite nanoparticle concentration / µg ml^{-1}	Mean of oxidation reaction potential (ORP) after 7 days	LSD value =6.44 Alpha = 0.05	LSD value =8.58 Alpha =0.01
0	227	A	a
40	174	B	b
80	171	B	b

Comparisons of the ORP means under pH effect showed that from alkaline pH to acidic, the ORP means had a upward trend at the measured times (zero time and after 7 days), and also the differences of ORP means under pH levels were significant (Table 5-29 and 30).

Table 5-29. Comparison of the ORP means at zero time under pH effect in liquid culture media of *Bradyrhizobium japonicum* Histic.

pH	Mean of oxidation reaction potential (ORP) at zero time	LSD value =7.93	LSD value =10.57
		Alpha =0.05	Alpha =0.01
4	300.5	A	a
5	272	B	b
6	228.5	C	c
7	201.6	D	d
8	85.17	E	e
9	46.08	F	f

Table 5-30. Comparison of the ORP means after 7 days under pH effect in liquid culture media of *Bradyrhizobium japonicum* Histic.

pH	Mean of oxidation reaction potential (ORP) after 7 days	LSD value =9.108	LSD value =12.14
		Alpha =0.05	Alpha =0.01
4	306	A	a
5	278.1	B	b
6	225.5	C	c
7	207.4	D	d
8	90.08	E	e
9	64.00	F	f

Comparisons of the ORP means under interaction effect between the nanoparticles and pH showed that 40 µg ml^{-1} of the nanoparticles at the different pH levels, prevent increasing the ORP than high concentration of the nanoparticles (80 µg ml^{-1}) (Tables 5-31and 32).

Table 5-31. Comparison of the ORP means at zero time under interaction effect between magnetite nanoparticle concentration and pH in liquid culture media of *Bradyrhizobium japonicum* Histic.

Interaction effect between magnetite nanoparticle concentration and pH	Mean of oxidation reaction potential (ORP) at zero time	LSD value =13.73	LSD value =18.31
		Alpha = 0.05	Alpha = 0.01
0-4	289.5	B	bc
0-5	272.5	C	cd
0-6	234	D	e
0-7	225	D	ef
0-8	104	G	h
0-9	73.75	H	i
40-4	308.5	A	a
40-5	273.3	C	cd
40-6	229.3	D	e
40-7	207.5	E	fg
40-8	99.25	G	h
40-9	66.50	H	ij
80-4	303.5	A	ab
80-5	270.3	C	d
80-6	222.3	D	ef
80-7	189.8	F	g
80-8	67	H	lj
80-9	51.75	I	j

Table 5-32. Comparison of the ORP means at zero time under interaction effect between magnetite nanoparticle concentration and pH in liquid culture media of *Bradyrhizobium japonicum* Histic.

Interaction effect between magnetite nanoparticle concentration and pH	Mean of oxidation reaction potential (ORP) after 7 days	LSD value =15.77	LSD value =21.02
		Alpha = 0.05	Alpha = 0.01
0-4	339	A	a
0-5	307.5	B	b
0-6	256.5	EF	de
0-7	236	G	e
0-8	126.8	J	i
0-9	93	K	j
40-4	286.8	CD	bc
40-5	255.3	F	de
40-6	209.5	H	fg
40-7	189	I	gh
40-8	76.75	L	j
40-9	26.25	N	l
80-4	292.3	BC	bc
80-5	271.5	DE	cd
80-6	210.5	H	f
80-7	179.8	I	h
80-8	52	M	k
80-9	19	N	l

5.4.8 Discussion

According to the above results, ORP increased during *Bradyrhizobium japonicum* Histic growth without nanoparticles (Figure 5-15), doubtless due to releasing ROS and RNS[15,81,232,241], while in the presence of the magnetite nanoparticles the secretions of *Bradyrhizobium* were presumably sequestered by the nanoparticles, hence the nanoparticles decreased the ORP.

Previous studies indicated that the *B. japonicum* has antioxidant defence (AD), which involves enzymatic activities (catalases, superoxide dismutases, peroxidases), and altogether, ROS, RNS and AD contribute to the redox balance, the modulation of which is probably crucial for physiological regulation [15,185,232]. Clearly, variation of pH could affect the enzymatic activities, and change the redox of the culture medium [12, 15, 22, 48, 74, 185, 232]. Because extreme conditions like pH acid or alkaline is an obstacle to the redox balance. Besides, survival and increasing bacterial growth depend on antioxidant defence activity in extreme conditions [48, 81, 185]. Thus any factor which can increased AD activity, increases survival and physiological indices. In this study treatments with nanoparticles could increase significantly viability of *B. japonicum* cells. Based on the results, one of nanoparticles' roles was buffering effect, and decreasing ORP in presence of the nanoparticles, which is an evidence for binding with ROS and RNS.

The nanoparticles as catalysts could convert superoxides to peroxides and oxygen molecules and then produced water molecules ($O_2^{\cdot -} + O_2^{\cdot -} + 2H^+ \rightarrow H_2O_2 + O_2$ and $H_2O_2 + 2H^+ \rightarrow 2H_2O$), and also they likely were able to absorb water molecules temporarily [30,185,194,252], and then they could prevent decreasing availabel oxygen during bacterial culture.

Although some constituents in the YMB like K_2HPO_4 are used to buffer variation of pH during growth, the results showed that it could not act effectively in the extreme conditions, while the nanoparticles were potent in the alkaline pH and they could apparent moderate extreme conditions for bacterial growth. In fact nanoparticles have capacity of preserving oxygen molecules by neutralization of the ROS and RNS [30,153,179,194,252]. Preserving optimum conditions like the a_w and

the ORP in the extreme pH by the nanoparticles could enhanced N_V, NG, GRC and decreased the MGT.

On the other hand application of a high concentration of magnetite nanoparticles has inverse effects on the optimum physiological indices of the bacterial growth. In presence of a high concentration of iron, bacteria release some proteins to capture it, thus countring any effect [27], but, which imposes an energetic burden. In addition, there are omnipresent negative effects of iron particles, dependent on other growth conditions like pH (for instance at low pH, iron catalyzes superoxide and hydrogen peroxide forming highly reactive (hence toxic) hydroxyl radicals [97]). Furthermore, a high concentration of nanoparticles may induce a high osmotic pressure in the liquid media, inducing plasmolysis, which obviously decreases the survival of bacterial cells. However, in order to clarify the effects of high concentration of the nanoparticles on the growth of bacteria more evidence and investigation is needed.

5.4.9 Conclusions

Poor *B. japonicum* growth under extreme condition can be revived by adding magnetite nanoparticles. The optimum concentration was 40 µg ml^{-1} of nanoparticles. Furthermore, useful enhancement of growth even at standard pH (6.8) could be obtained by adding nanoparticles. Evidence from water activity and ORP suggested that sequestration of secreted ROS is the most significant effect of the nanoparticles. However, in order to verify this proposition more investigation is necessary.

5.5 Effects of salinity and magnetite nanparticles on the *Bradyrhizobium* growth rate

Note that this experiment was carried out in two groups of nanoparticles concentrations.

5.5.1 Effects of magnetite nanoparticle concentration (0, 40, 80 µg ml^{-1}) and salinity (0, 600, 1200, ..., 4800 µS cm^{-1}) on growth indices of the *Bradyrhizobium* growth rate

5.5.1.1 Mean generation time (MGT)

Analysis of variation (Table 5-33) showed that growth indices (MGT, GRC and NG) were significantly affected by magnetite nanoparticle concentration, salinity and their interaction effects. The results showed that 40 µg ml^{-1} of the nanoparticles had a positive effect on the mean generation time (MGT); this concentration decreased the mean of the MGT compared to the other treatments (Figure 5-12). Increasing salt concentration in the liquid culture media increased the MGT; i.e., a negative response of bacterial growth (Figure 5-12). The results of interaction effects analysis between nanoparticles and salinity showed that when 40 µg ml^{-1} of the nanoparticles was used, there was a decreased inhibitory effect of salinity on the MGT (Figure 5-12). However, high a concentration of the nanoparticles was not able to decrease inhibitory effect of salinity on the MGT.

Table 5-33. Means squares from analysis of variation of the growth indices of the *Bradyrhizobium japonicum* Histic.

Source of Variation	df	Mean squares		
		MGT/ h	GRC / h^{-1}	NG
Blocks	3	0.3	0.0003	0.6
Nanoparticles† (N_P)	2	30 **	0.004**	70.5**
Salinity (S)	8	3.4**	0.0004*	7.1**
N_P × S	16	1.5**	0.0004*	5.1**
Residual	78	0.2	0.0002	1.3

† Magnetite nanoparticles (0, 40, 80 µg ml^{-1}); *, ** *F* test indicated significance at $P< 0.05$ and $P< 0.01$, respectively.

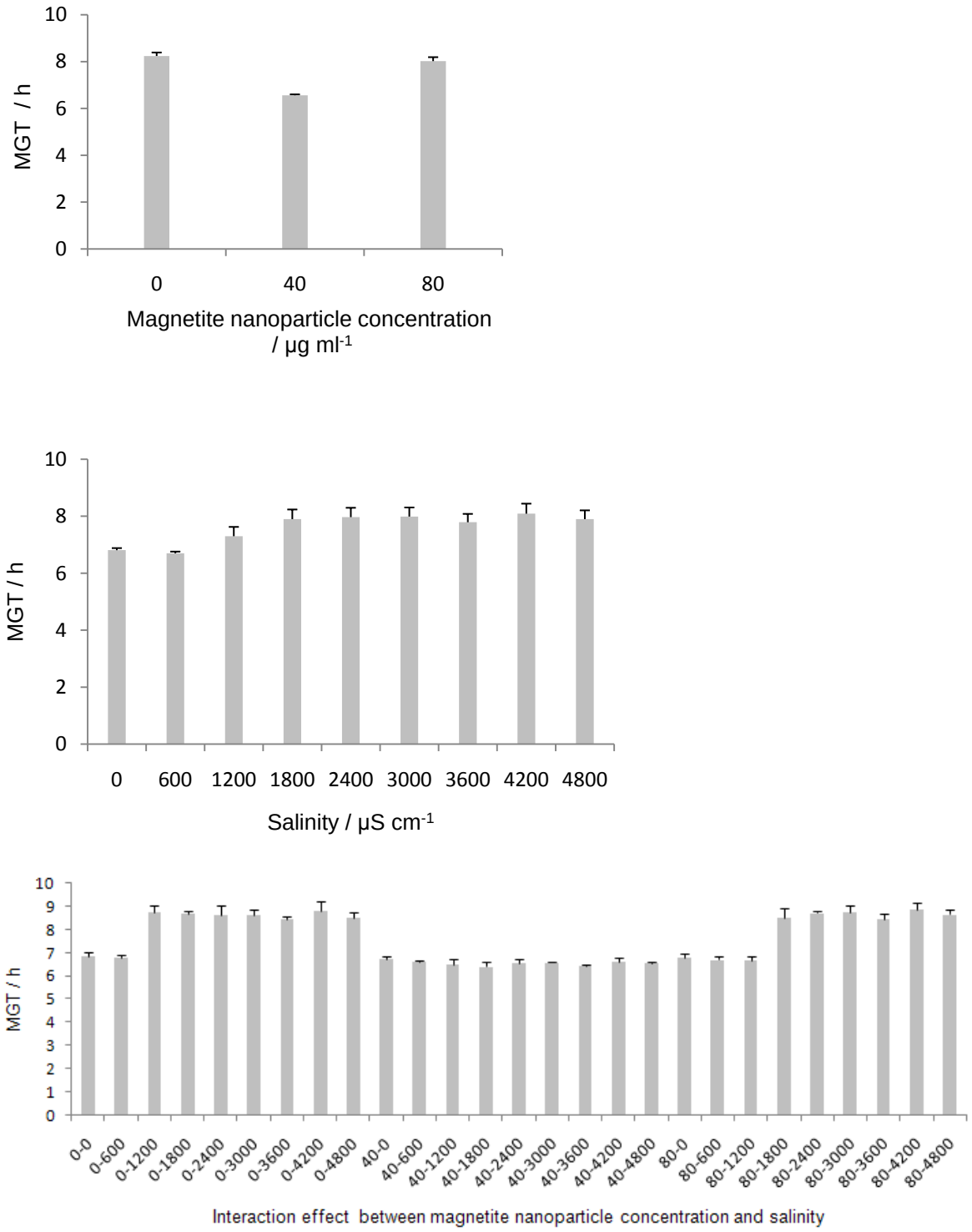

Figure 5-12. Effect of (top) magnetite nanoparticle concentration (average over all salt concentrations), (centre) salinity (average over all nanoparticle concentrations), and (bottom) their interaction (labelled as $N_{P\ conc.}$-salinity) on the mean generation time (MGT) of *Bradyrhizobium japonicum* Histic. Error bars show standard error.

5.5.1.2 Number of generations (NG)

The NG was significantly affected by nanoparticles in the liquid media at $P<0.01$(Table 5-33). The results showed that 40 µg ml^{-1} of the nanoparticles increased NG by about 14 times (Figure 5-13). The NG was significantly affected by salinity conditions at $P<0.01$ (Table 5-33). Increasing salt concentrations in liquid culture media had a negative effect on the NG (Figure 5-13).

However, ananlysis of interaction effects between the nanoparticles and salinity showed that when 40 µg ml^{-1} of the nanoparticles was used, the NG increased under some salinity conditions (Figure 5-13).

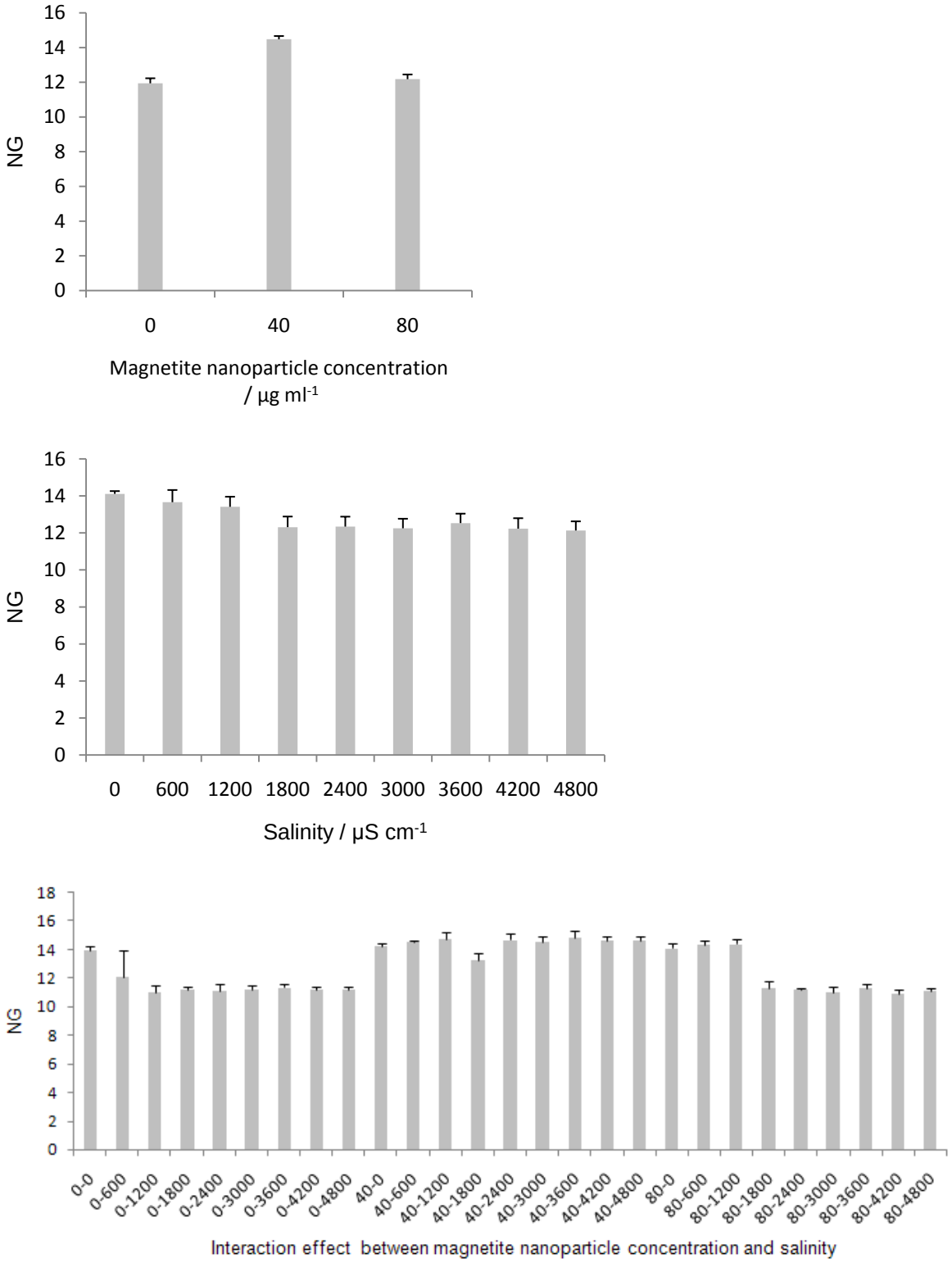

Figure 5-13. Effect of (top) magnetite nanoparticle concentration (average over all salt concentrations), (centre) salinity (average over all nanoparticle concentrations), and (bottom) their interaction (labelled as $N_{P\ conc.}$-salinity) on the number of generations (NG) of *Bradyrhizobium japonicum* Histic. Error bars show standard error.

5.5.1.3 Growth rate constant (GRC)

The GRC was affected by the nanoparticles, salinity and their interaction at $P<0.01$ (Table 5-33). The results showed that the nanoparticles had a positive effect on the GRC (Figure 5-14). Salinity decreased the GRC (a negative effect) (Figure 5-14). However, when 40 µg ml^{-1} of the nanoparticles was used, the GRC increased under some salinity conditions (Figure 5-14).

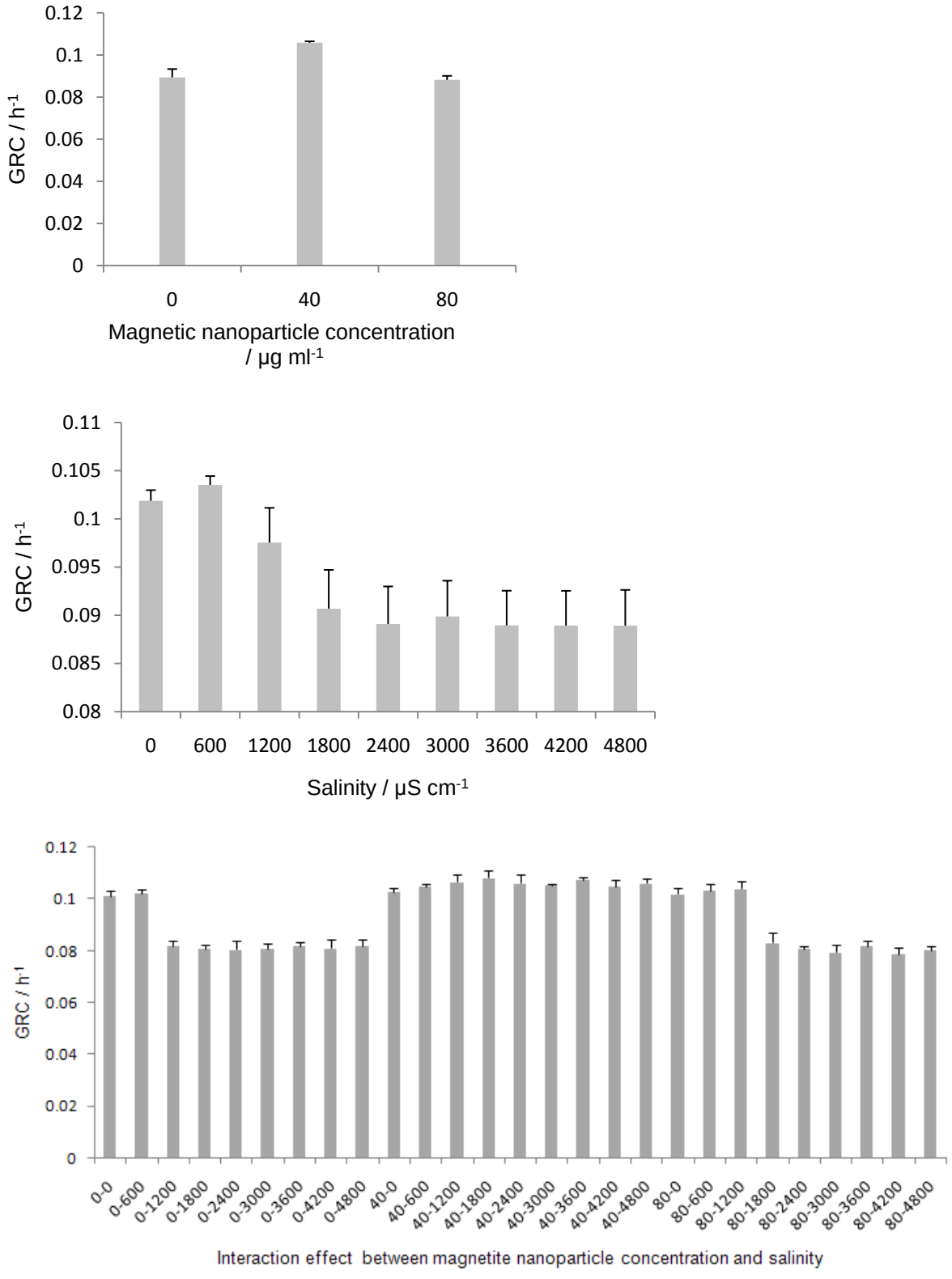

Figure 5-14. Effect of (top) magnetite nanoparticle concentration (average all salt concentrations), (centre) salinity (average over all nanoparticle concentrations), and (bottom) their interaction (labelled as $N_{P\ conc.}$-salinity) on the growth rate constant (GRC) of the *Bradyrhizobium japonicum* Histic. Error bars show standard error.

5.5.1.4 Comparison of the growth indices means (MGT, NG, and GRC) of *Bradyrhizobium* growth rate under magnetite nanoparticle concentration (0, 40, 80 µg ml^{-1}) and salinity (0, 600, 1200, ..., 4800 µS cm^{-1})

Comparisons of the MGT means under the nanoparticles effects showed that the lowest MGT mean was obtained by 40 µg ml^{-1} of the nanoparticles, while the greatest MGT was obtained by the control treatment (Table 5-34). Comparisons of salinity effects on the MGT showed that increasing salinity concentration in liquid culture media increased the MGT means compared to the control treatment (Table 5-35). The significant interaction was due to the salinity having no effect on MGT when 40 µg ml^{-1} of the nanoparticles were present compared to the control and 80 µg ml^{-1} of the nanoparticles (Table 5-36).

Table 5-34. Comparison of the MGT means of *Bradyrhizobium japonicum* Histic under magnetite nanoparticle concentration effect.

Magnetite nanoparticle concentration / µg ml^{-1}	Mean of mean generation time (MGT) / h	LSD value = 0.1963	LSD value = 0.2603
		Alpha = 0.05	Alpha = 0.01
0	8.237	A	a
40	6.560	C	b
80	8.018	B	a

Table 5-35. Comparison of the MGT means of *Bradyrhizobium japonicum* Histic under salinity effect.

Salinity / µS cm^{-1}	Mean of mean generation time (MGT) / h	LSD value = 0.3400	LSD value = 0.4509
		Alpha = 0.05	Alpha = 0.01
0	6.812	C	c
600	6.702	C	c
1200	7.306	B	b
1800	7.883	A	a
2400	7.974	A	a
3000	7.987	A	a
3600	7.793	A	a
4200	7.809	A	a
4800	7.901	A	a

Table 5-36. Comparison of the MGT means of *Bradyrhizobium japonicum* Histic under interaction effect between magnetite nanoparticle concentration and salinity.

Interaction effect between magnetite nanoparticle concentration and salinity	Mean of mean generation time (MGT) / h	LSD value = 0.5889	LSD value = 0.781
		Alpha = 0.05	Alpha = 0.01
0-0	6.874	B	b
0-600	6.785	B	b
0-1200	8.726	A	a
0-1800	8.712	A	a
0-2400	8.654	A	a
0-3000	8.617	A	a
0-3600	8.458	A	a
0-4200	8.800	A	a
0-4800	8.510	A	a
40-0	6.75	B	b
40-600	6.612	B	b
40-1200	6.517	B	b
40-1800	6.421	B	b
40-2400	6.549	B	b
40-3000	6.587	B	b
40-3600	6.452	B	b
40-4200	6.615	B	b
40-4800	6.538	B	b
80-0	6.812	B	b
80-600	6.711	B	b
80-1200	6.675	B	b
80-1800	8.516	A	a
80-2400	8.717	A	a
80-3000	8.756	A	a
80-3600	8.470	A	a
80-4200	8.852	A	a
80-4800	8.655	A	a

5.5.1.4.1 Comparisons of number of generations means (NG)

Comparisons of the NG means under the nanoparticles effects showed that the greatest NG was obtained by 40 µg ml^{-1} of the nanoparticles, and also, the differences of the NG means between the control and 80 µg ml^{-1} of the nanoparticles were not significant (Table 5-37). However, comparisons of salinity conditions on the NG means showed that the greatest NG mean was occurred by the control treatment and 600 and 1200 µS cm^{-1} of salt concentrations (Table 5-38). The significant interaction effects, was due to there bears no effect of increases salinity when 40 µg ml^{-1} of the nanoparticles were present.

Table 5-37. Comparison of the NG means of *Bradyrhizobium japonicum* Histic under magnetite nanoparticle concentration effect.

Magnetite nanoparticle concentration / µg ml^{-1}	Mean of number of generations (NG)	LSD value = 0.5348	LSD value = 0.7093
		Alpha = 0.05	Alpha = 0.01
0	11.94	B	b
40	14.48	A	A
80	12.18	B	b

Table 5-38. Comparison of the NG means of *Bradyrhizobium japonicum* Histic under salinity effect.

Salinity / µS cm^{-1}	Mean of number of generations (NG)	LSD value = 0.9263	LSD value = 1.229
		Alpha = 0.05	Alpha = 0.01
0	14.11	A	a
600	13.66	A	ab
1200	13.41	AB	abc
1800	12.31	C	d
2400	12.34	C	cd
3000	12.25	C	cd
3600	12.53	BC	bcd
4200	12.23	C	cd
4800	12.13	BCD	cd

Table 5-39. Comparison of the NG means of *Bradyrhizobium japonicum* Histic under interaction effect between magnetite nanoparticle concentration and salinity.

Interaction effect between magnetite nanoparticle concentration and salinity	Mean of number of generations (NG)	LSD value = 1.604	LSD value = 2.128
		Alpha = 0.05	Alpha = 0.01
0-0	13.99	A	ab
0-600	12.12	BC	bcd
0-1200	11.05	C	d
0-1800	11.19	C	cd
0-2400	11.15	C	d
0-3000	11.17	C	d
0-3600	11.36	C	cd
0-4200	11.20	C	cd
0-4800	11.19	C	cd
40-0	14.23	A	ab
40-600	14.52	A	a
40-1200	14.77	A	a
40-1800	14.7	A	a
40-2400	14.7	A	a
40-3000	14.57	A	a
40-3600	14.88	A	a
40-4200	14.61	A	a
40-4800	14.69	A	a
80-0	14.11	A	ab
80-600	14.33	A	a
80-1200	14.41	A	a
80-1800	11.31	C	cd
80-2400	11.17	C	d
80-3000	11	C	d
80-3600	11.35	C	cd
80-4200	10.88	C	d
80-4800	11.11	C	d

5.5.1.4.2 Comparisons of growth rate constant means (GRC)

Comparisons of the GRC means under the nanoparticles effect showed that the greast GRC mean was obtained by 40 µg ml^{-1} of the nanoparticles, while the lowest of GRC mean was obtained by 80 µg ml^{-1} of the nanoparticles (Table 5-40). Comparisons of salinity conditions on the GRC means showed that the greatest of GRC was obtained by control treatment (Table 5-41). The greatest GRC mean was obtained under interaction effects between 40 µg ml^{-1} of the nanoparticles and different salt concentrations compared to the other treatments (Table 5-42).

Table 5-40. Comparison of the GRC means of *Bradyrhizobium japonicum* Histic under magnetite nanoparticle concentration effect.

Magnetite nanoparticle concentration / µg ml^{-1}	Mean of growth rate constant (GRC) / h^{-1}	LSD value = 0.0065	LSD value = 0.0088
		Alpha = 0.05	Alpha = 0.01
0	0.08936	B	b
40	0.10581	A	A
80	0.08814	B	b

Table 5-41. Comparison of the GRC means of *Bradyrhizobium japonicum* Histic under salinity effect.

Salinity / µS cm^{-1}	Mean of growth rate constant (GRC) / h^{-1}	LSD value = 0.0011	LSD value = 0.0015
		Alpha = 0.05	Alpha = 0.01
0	0.10388	A	a
600	0.10352	A	a
1200	0.09754	AB	ab
1800	0.09069	B	ab
2400	0.08909	B	ab
3000	0.08985	B	ab
3600	0.08894	B	ab
4200	0.088935	B	ab
4800	0.088931	B	ab

Table 5-42. Comparison of the GRC means of *Bradyrhizobium japonicum* Histic under interaction effect between nanoparticle and salinity.

Interaction effect between magnetite nanoparticle concentration and salinity	Mean of growth rate constant (GRC) / h^{-1}	LSD value = 0.0019	LSD value = 0.026
		Alpha = 0.05	Alpha = 0.01
0-0	0.10099	ABC	abcdefgh
0-600	0.10224	AB	abcdefgh
0-1200	0.08196	CD	cdefgh
0-1800	0.08079	D	efgh
0-2400	0.08052	D	efgh
0-3000	0.08063	D	efgh
0-3600	0.082	CD	cdefgh
0-4200	0.081	CD	defgh
0-4800	0.0817	CD	defgh
40-0	0.10278	A	abcdefgh
40-600	0.10487	A	abcdefg
40-1200	0.10663	A	abcd
40-1800	0.10818	A	ab
40-2400	0.10612	A	abcde
40-3000	0.10524	A	abcdef
40-3600	0.10746	A	abc
40-4200	0.10497	A	abcdefg
40-4800	0.10609	A	abcde
80-0	0.1019	AB	abcdefgh
80-600	0.10344	A	abcdefgh
80-1200	0.10404	A	abcdefgh
80-1800	0.08309	BCD	bcdefgh
80-2400	0.08064	D	efgh
80-3000	0.07943	D	gh
80-3600	0.08196	CD	cdefgh
80-4200	0.07857	D	h
80-4800	0.0802	D	fgh

5.5.1.5 Response of water activity (a_w) to magnetite nanoparticle concentration (0, 40, 80 µg ml^{-1}) and salinity (0, 600, 1200, ..., 4800 µS cm^{-1})

The a_w was affected by magnetite nanoparticle concentration, salinity and their interaction effects at zero time and after 7 days in culture medium (Table 5-43). The results showed that with 40 µg ml^{-1} of the nanoparticles after 7 days the a_w was greater compared to the other treatments (Figure 5-15). In contrast, a high concentration (80 µg ml^{-1}) of nanoparticles decreased the a_w. However, interaction effect between nanoparticle and the a_w showed that these nanoparticles could decrease salinity effect on the a_w. Furthermore response of the a_w to salt concentrations from 600 to 3600 µS cm^{-1} slightly decreased (Figure 5-15).

Table 5-43. Mean squares from analysis of variation of the water activity (a_w).

Source of Variation	df	Mean squares	
		Zero time	After 7 days
Blocks	3	0.12	0.53
Nanoparticles† (N_P)	2	0.61**	0.21**
Salinity (S)	8	0.51**	0.17**
N_P × S	16	32**	0.11**
Residual	78	0.068	0.27

† Magnetite nanoparticles (0, 40, 80 µg ml^{-1}); *, ** *F* test indicated significance at $P< 0.05$ and $P< 0.01$, respectively.

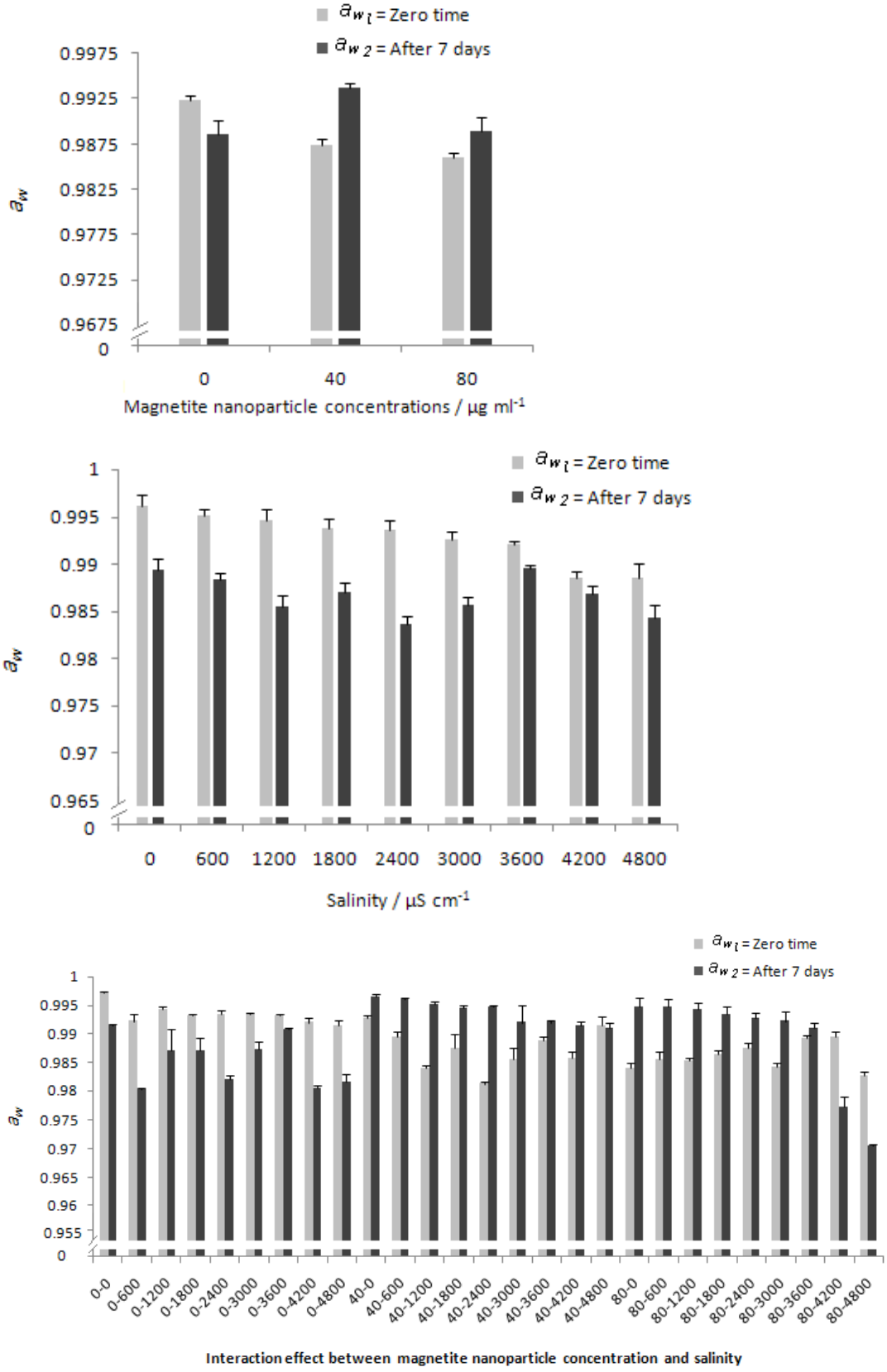

Figure 5-15. Response of water activity (a_w) to (top) the nanoparticles (average over all salt concentrations), (centre) salinity (average over all nanoparticle concentrations) and (bottom) their interaction (labelled as $N_{P\ conc.}$-salinity); gray bars and black bars indicate measured times at zero time and after 7 days of bacterial growth, respectively; error bars show standard error.

5.5.1.5.1 Comparisons of water activity (a_w) means under magentite nanoparticle concentration (0, 40, 80 µg ml^{-1}) and salinity (0, 600, 1200, ..., 4800 µS cm^{-1})

Comparisons of the water activity means under effect of nanoparticles showed that at zero time, control treatment had the greatest of water activity mean, while after 7days, both the control treatment and 40 µg ml^{-1} of the nanoparticles had the greatest of water activity mean (Table 5-44 and 45). Comparisons of the water activity means under salinity conditions showed that the water activity means were decreased after 7 days (Table 5-46 and 47). However, comparisons of the water activity means under interaction effects between the nanoparticles and salt concentrations showed that the water activity means were increased by interaction effect between 40 µg ml^{-1} of the nanoparticles and 600 to 2400 µS cm^{-1} of salt concentrations (Table 5-48 and 49).

Table 5-44. Comparison of water activity means at zero time under magnetite nanoparticle concentration effect in liquid culture media of *Bradyrhizobium japonicum* Histic.

Magnetite nanoparticle concentration / µg ml^{-1}	Mean of water activity (a_w) at zero time	LSD value = 0.00122	LSD value = 0.00162
		Alpha = 0.05	Alpha = 0.01
0	0.98865	A	a
40	0.98738	B	ab
80	0.98605	C	b

Table 5-45. Comparison of water activity means after 7 days under magnetite nanoparticle concentration effect in liquid culture media of *Bradyrhizobium japonicum* Histic.

Magnetite nanoparticle concentration / µg ml^{-1}	Mean of water activity (a_w) after 7 days	LSD value = 0.00245	LSD value = 0.00325
		Alpha = 0.05	Alpha = 0.01
0	0.99231	A	a
40	0.99372	A	a
80	0.98900	B	b

Table 5-46. Comparison of water activity means at zero time under salinity effect in liquid culture media of *Bradyrhizobium japonicum* Histic.

Salinity / µS cm^{-1}	Mean of water activity (a_w) at zero time	LSD value = 0.00211	LSD value = 0.00281
		Alpha = 0.05	Alpha = 0.01
0	0.989417	A	a
600	0.988417	AB	ab
1200	0.98545	CD	cd
1800	0.987	BC	abc
2400	0.983583	D	d
3000	0.985667	CD	bcd
3600	0.989583	A	a
4200	0.988583	AB	a
4800	0.988583	AB	a

Table 5-47. Comparison of water activity means after 7 days under salinity effect in liquid culture media of *Bradyrhizobium japonicum* Histic.

Salinity / µS cm^{-1}	Mean of water activity (a_w) after 7 days	LSD value =0.0042	LSD value = 0.0056
		Alpha = 0.05	Alpha = 0.01
0	0.996167	A	a
600	0.991	BC	ab
1200	0.994583	AB	a
1800	0.99375	AB	a
2400	0.993667	AB	a
3000	0.992583	AB	a
3600	0.992083	AB	ab
4200	0.986917	CD	bc
4800	0.984333	D	c

Table 5-48. Comparison of water activity means at zero time under interaction effect between magnetite nanoparticle concentration and salinity in liquid culture media of *Bradyrhizobium japonicum* Histic.

Interaction effect between magnetite nanoparticle concentration and salinity	Mean of water activity (a_w) at zero time	LSD value = 0.00366	LSD value = 0.00486
		Alpha = 0.05	Alpha = 0.01
0-0	0.99725	A	a
0-600	0.99225	BCD	bcd
0-1200	0.99425	AB	ab
0-1800	0.99325	B	abc
0-2400	0.9935	B	abc
0-3000	0.9935	B	abc
0-3600	0.99325	B	abc
0-4200	0.992	BCD	bcd
0-4800	0.9915	BCD	bcd
40-0	0.99275	BC	abc
40-600	0.98950	CDE	bcde
40-1200	0.98400	HIJ	fgh
40-1800	0.98750	EFGH	defg
40-2400	0.98125	J	h
40-3000	0.98550	GHI	efgh
40-3600	0.98875	DEFG	cdef
40-4200	0.98575	FGHI	efgh
40-4800	0.99150	BCD	bcd
80-0	0.98400	HIJ	fgh
80-600	0.98550	GHI	efgh
80-1200	0.98525	GHI	efgh
80-1800	0.98650	EFGH	efg
80-2400	0.98750	EFGH	defg
80-3000	0.98425	HIJ	fgh
80-3600	0.98925	CDEF	cde
80-4200	0.98950	CDE	bcde
80-4800	0.98275	IJ	gh

Table 5-49. Comparison of water activity means after 7 days under interaction effect between magnetite nanoparticle concentration and salinity in liquid culture media of *Bradyrhizobium japonicum* Histic.

Interaction effect between magnetite nanoparticle concentration and salinity	Mean of water activity (a_w) after 7 days	LSD value = 0.0055	LSD value = 0.0073
		Alpha = 0.05	Alpha = 0.01
0-0	0.9915	ABC	ab
0-600	0.98025	DE	cde
0-1200	0.9871	CD	abc
0-1800	0.987	CD	abcd
0-2400	0.982	DE	bcd
0-3000	0.98725	BCD	abc
0-3600	0.99075	ABC	ab
0-4200	0.9805	DE	cd
0-4800	0.9805	DE	cd
40-0	0.99650	A	a
40-600	0.99600	A	a
40-1200	0.99525	A	a
40-1800	0.99450	AB	a
40-2400	0.99475	A	a
40-3000	0.99200	ABC	a
40-3600	0.99200	ABC	a
40-4200	0.99150	ABC	ab
40-4800	0.99100	ABC	ab
80-0	0.99475	A	a
80-600	0.99475	A	a
80-1200	0.99425	ABC	a
80-1800	0.99350	ABC	a
80-2400	0.99275	ABC	a
80-3000	0.99225	ABC	a
80-3600	0.99100	ABC	ab
80-4200	0.97725	EF	de
80-4800	0.97050	F	e

5.5.1.6 Response of oxidation reaction potential (ORP) to magnetite nanoparticle concentration (0, 40, 80 µg ml^{-1}) and salinity (0, 600, 1200, ..., 4800 µS cm^{-1})

The ORP was affected by nanoparticles, salinity, and their interaction effects (Table 5-50). Results showed that 40 µg ml^{-1} could prevent increasing ORP more than other treatments (Figure 5-16), while the control treatment increased the ORP. At zero time, the ORP was significantly affected by salinity, and also salinity had an additive effect on the ORP as a negative response. Although the ORP means increased after 7 days (Figure 5-16), but the increases were not significant (Table 5-50).

Table 5-50. Mean squares from analysis of variation of the oxidation reaction potential (ORP).

		Mean squares	
Source of Variation	df	Zero time	After 7 days
Blocks	3	480	211
Nanoparticles† (N_P)	2	1799**	10991.2**
Salinity (S)	8	353**	$161.1^{n.s.}$
$N_P \times S$	16	$138^{n.s.}$	340*
Residual	78	90	117

† Magnetite nanoparticles (0, 40, 80 µg ml^{-1}), *, ** *F* test indicated significance at $P< 0.05$ and $P< 0.01$, respectively; $^{n.s.}$ indicates a nonsignificant result.

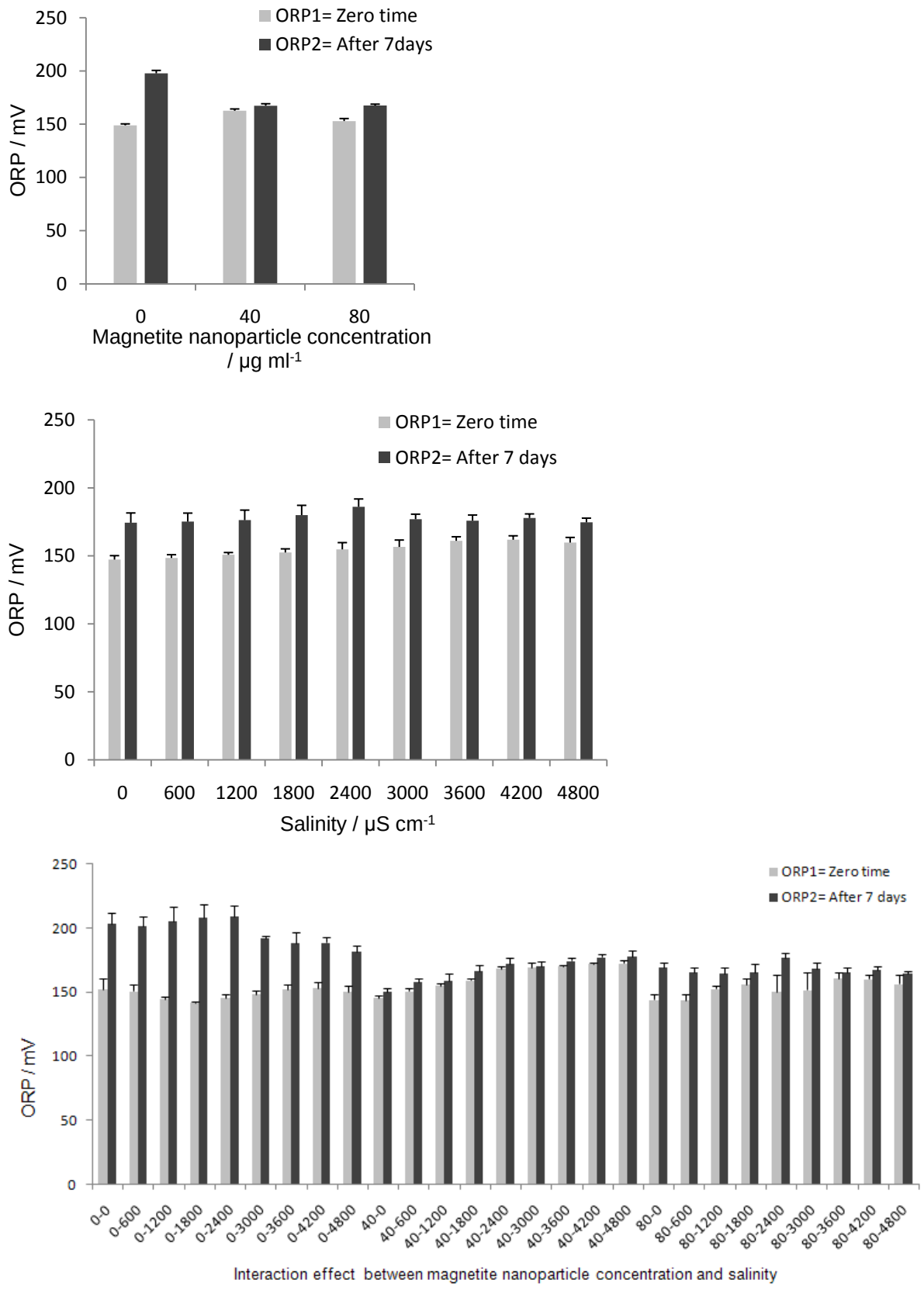

Figure 5-16. Response of the ORP to (top) the nanoparticles (average over all salt concentrations) and (centre) the salinity (average over all nanoparticle concentrations) and (bottom) their interaction (labelled as $N_{P\ conc.}$-salinity); gray bars and black bars indicate measured times at zero time and after 7 days of bacterial growth, respectively. Error bars show standard error.

5.5.1.6.1 Comparison of oxidation reaction potential (ORP) means under magnetite nanoparticle concentration (0, 40, 80 µg ml^{-1}) and salinity (0, 600, 1200, ..., 4800 µS cm^{-1})

Comparisons of the ORP means under the nanoparticles showed that by 40 µg ml^{-1} of the nanoparticles, the greatest of ORP mean obtained at zero time, while after 7 days, the greatest of ORP mean obtained by control treatment (Table 5-51, 52). The results of the salinity effect on the ORP means showed that control treatment had the lowest of ORP mean at zero time, after 7 days, although salt concentrations and control treatments increased the ORP mean, but their difference of means were not high and they were ranked in a same level (Tables 5-53 and 54). However, comparisons of the ORP means under interaction effect between the nanoparticles and salinity showed that at zero time were not significant (Table 5-50). After 7 days 40 µg ml^{-1} of the nanoparticles in the different salt concentrations could more prevent increasing of the ORP than high concentration of the nanoparticles (80 µg ml^{-1}), and also the greatest of ORP occurred by the control treatment (Table 5-55).

Table 5-51. Comparison of the ORP means at zero time under magnetite nanoparticle concentration effect in liquid culture media of *Bradyrhizobium japonicum* Histic.

Magnetite nanoparticle concentration / µg ml^{-1}	Mean of oxidation reaction potential (ORP) at zero time.	LSD value = 4.451	LSD value = 5.903
		Alpha = 0.05	Alpha = 0.01
0	148.9	B	B
40	162.6	A	A
80	152.9	B	B

Table 5-52. Comparison of the ORP means after 7 days under magnetite nanoparticle concentration effect in liquid culture media of *Bradyrhizobium japonicum* Histic.

Magnetite nanoparticle concentration / µg ml^{-1}	Mean of oxidation reaction potential (ORP) After 7 days	LSD value = 5.067	LSD value = 6.72
		Alpha = 0.05	Alpha = 0.01
0	197.7	A	a
40	167.3	B	b
80	167.5	B	b

Table 5-53. Comparison of the ORP means at zero time under salinity effect in liquid culture media of *Bradyrhizobium japonicum* Histic.

Salinity / µS cm^{-1}	Mean of oxidation reaction potential (ORP) at zero time.	LSD value = 7.709	LSD value = 10.22
		Alpha = 0.05	Alpha = 0.01
0	147.3	D	c
600	148.5	D	c
1200	150.8	CD	bc
1800	152.5	BCD	abc
2400	154.8	ABCD	abc
3000	156.6	ABC	abc
3600	161.1	A	a
4200	161.9	A	a
4800	159.8	AB	ab

Table 5-54. Comparison of the ORP means after 7 days under salinity effect in liquid culture media of *Bradyrhizobium japonicum* Histic.

Salinity / µS cm^{-1}	Mean of oxidation reaction potential (ORP) After 7 days	LSD value = 15.20
		Alpha = 0.05
0	174.4	A
600	175.2	A
1200	176.3	A
1800	180	A
2400	186.1	A
3000	177	A
3600	175.9	A
4200	177.9	A
4800	174.7	A

Table 5-55. Comparison of the ORP means after 7 days under interaction effect between magnetite nanoparticle concentration and pH in liquid culture media of *Bradyrhizobium japonicum* Histic..

Interaction effect between magnetite nanoparticle concentration and salinity	Mean of oxidation reaction potential (ORP) After 7 days	LSD value = 15.20	LSD value = 20.16
		Alpha = 0.05	Alpha = 0.01
0-0	203.8	ABC	abc
0-600	201.8	ABCD	abcd
0-1200	205.3	AB	abc
0-1800	208.5	A	ab
0-2400	209	A	a
0-3000	192	BCDE	abcde
0-3600	188	DEF	cdefg
0-4200	188.8	CDEF	bcdef
0-4800	182	EFG	defgh
40-0	150.3	J	j
40-600	158	IJ	ij
40-1200	158.8	IJ	ij
40-1800	166.3	HI	hij
40-2400	172.3	GHI	efghi
40-3000	170.5	GHI	fghi
40-3600	174.5	FGH	efghi
40-4200	177.5	EFGH	efghi
40-4800	177.8	EFGH	efghi
80-0	169.3	GHI	fghij
80-600	165.8	HI	hij
80-1200	164.8	HIJ	hij
80-1800	165.3	HIJ	hij
80-2400	177	EFGH	efghi
80-3000	168.5	GHI	ghij
80-3600	165.3	HIJ	hij
80-4200	167.5	GHI	hij
80-4800	164.3	HIJ	hij

5.5.2 Effects of magnetite nanoparticle concentration (0, 20, 60 µg ml^{-1}) and salinity (0, 600, 1200, ..., 4800 µS cm^{-1}) on growth indices of the *Bradyrhizobium* growth rate

5.5.2.1 Mean generation time (MGT)

Analysis of variation (Table 5-56) showed that growth indices (MGT, GRC and NG) were significantly affected by magnetite nanoparticles concentration, salinity and their interaction effects. The results showed that 20 µg ml^{-1} of the nanoparticles decreased the mean of MGT (Figure 5-17). Except the control treatment and 600 µS cm^{-1} of salt conentrations, other salt concentrations increased the MGT means (a negative effect) (Figure 5-17). The results of interaction effects between nanoparticle and salinity conditions showed that when 20 µg ml^{-1} of the nanoparticles was used with 600 to 2400 µS cm^{-1} of salinity concentrations, decreased inhibitory effects of salinity on the MGT (Figure 5-17). However, only, 60 µg ml^{-1} of the nanoparticles was able to decrease the average of MGT with 600 to 2400 µS cm^{-1} of salinity (Figure 5-17).

Table 5-56. Mean squares from analysis of variation of the growth indices of the *Bradyrhizobium japonicum* Histic.

Source of variation	df	Mean squares		
		MGT / h	GRC / h^{-1}	NG
Blocks	3	0.26	0.00003	1.46
Nanoparticles† (N_P)	2	19.25**	0.00314**	44.15**
Salinity (S)	8	4.36**	0.00083**	10.12**
N_P × S	16	1.18**	0.00018**	6.81**
Residual	78	0.39	0.00007	2.08

† Magnetite nanoparticles (0, 20, 60 µg ml^{-1});*, ***F* test indicated significance at *P*< 0.01.

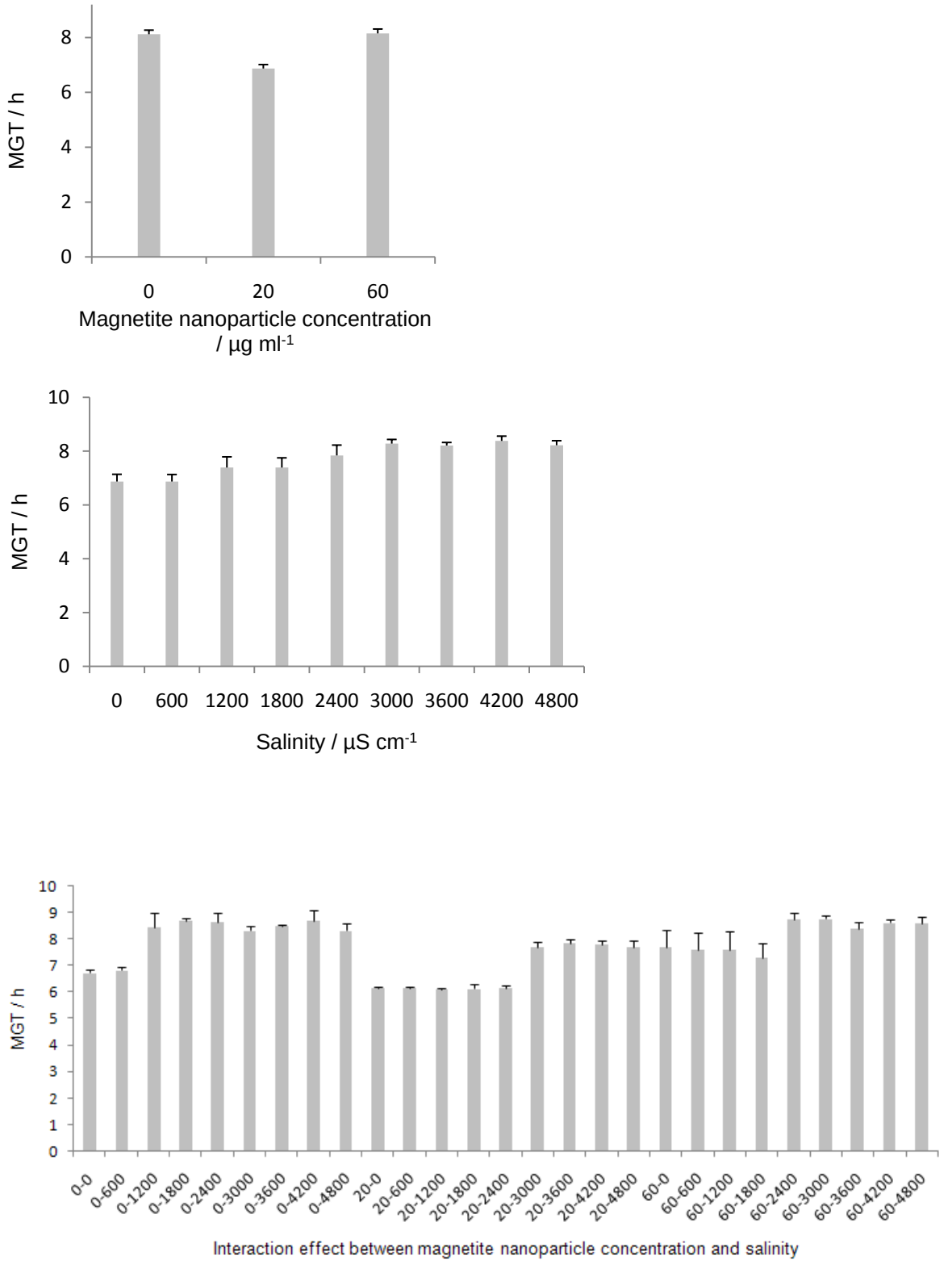

Figure 5-17. Effect of (top) magnetite nanoparticle concentration (average over all salt concentrations), (centre) salinity (average over all nanoparticle concentrations), and (bottom) their interaction (labelled as $N_{P\ conc.}$-salinity) on the mean generation time (MGT) of *Bradyrhizobium japonicum* Histic. Error bars show standard error.

5.5.2.2 Number of generations (NG)

The NG was significantly affected by nanoparticles, salinity and their interaction (Table 5-56). The results showed that 20 µg ml^{-1} of the nanoparticles increased mean of NG (Figure 5-18). Decrease in the NG mean was a negative response of bacterial growth due to increasing salinity (Figure 5-18). However, interaction effects between the nanoparticles and salinity showed that the NG increased in presence of the 20 µg ml^{-1} (the nanoparticles) and 600 to 2400 µS cm^{-1} of salinity concentrations, (Figure 5-18).

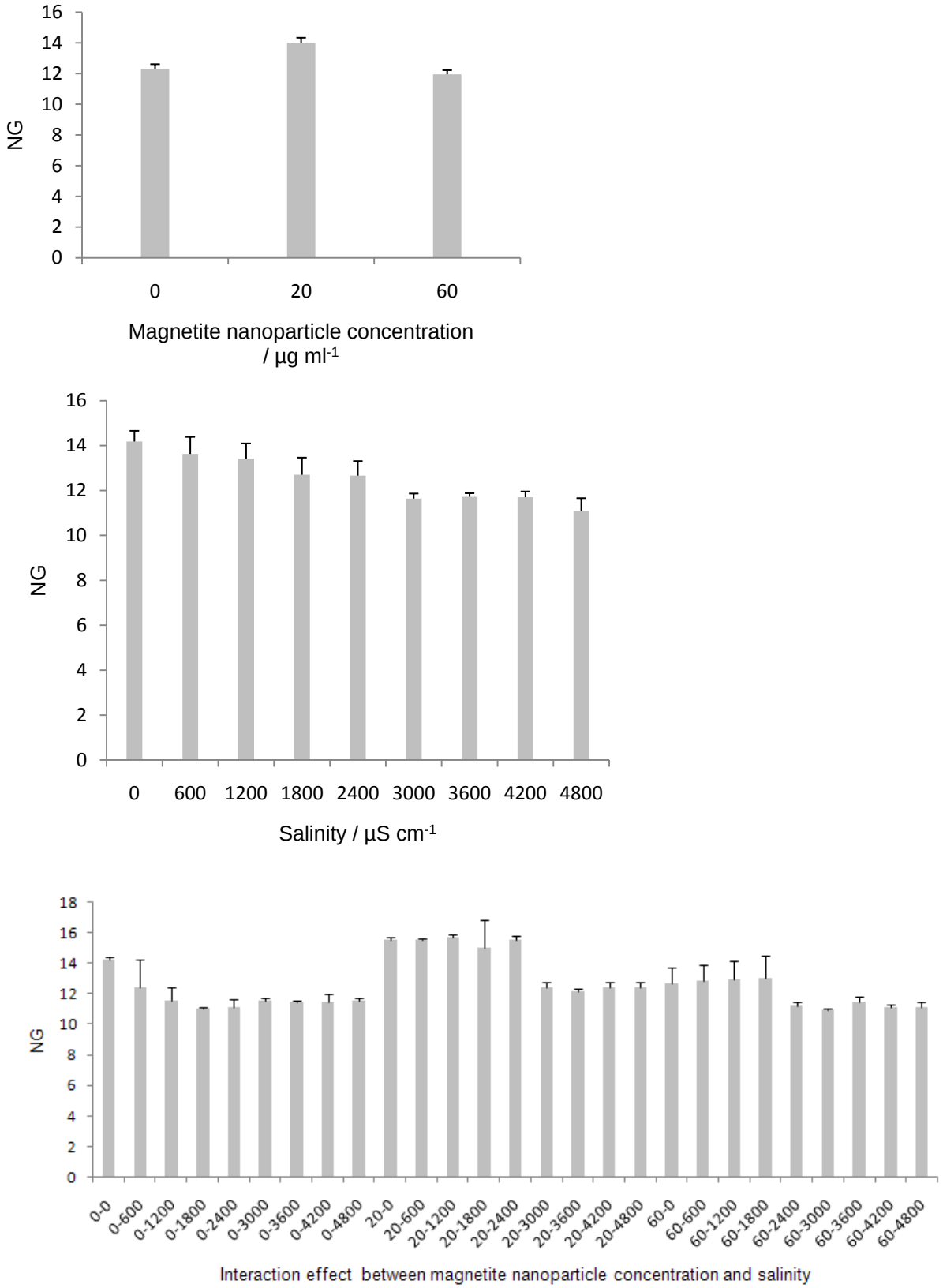

Figure 5-18. Effect of (top) magnetite nanoparticle concentration (average over all salt concentrations), (centre) salinity (average over all nanoparticle concentrations), and (bottom) their interaction (labelled as $N_{P\ conc.}$-salinity) on the number of generations (NG) of *Bradyrhizobium japonicum* Histic. Error bars show standard error.

5.5.2.3 Growth rate constant (GRC)

The GRC was affected by the nanoparticles, salinity and their interaction effects (Table 5-56). The results showed that the GRC means increased by 20 µg ml^{-1} of the nanoparticles (a positive effect) (Figure 5-19). Salinity decreased the GRC means (Figure 5-19). However, by 20 µg ml^{-1} of the nanoparticles with 600 to 2400 µS cm^{-1} of salt concentrations, the GRC increased (Figure 5-19).

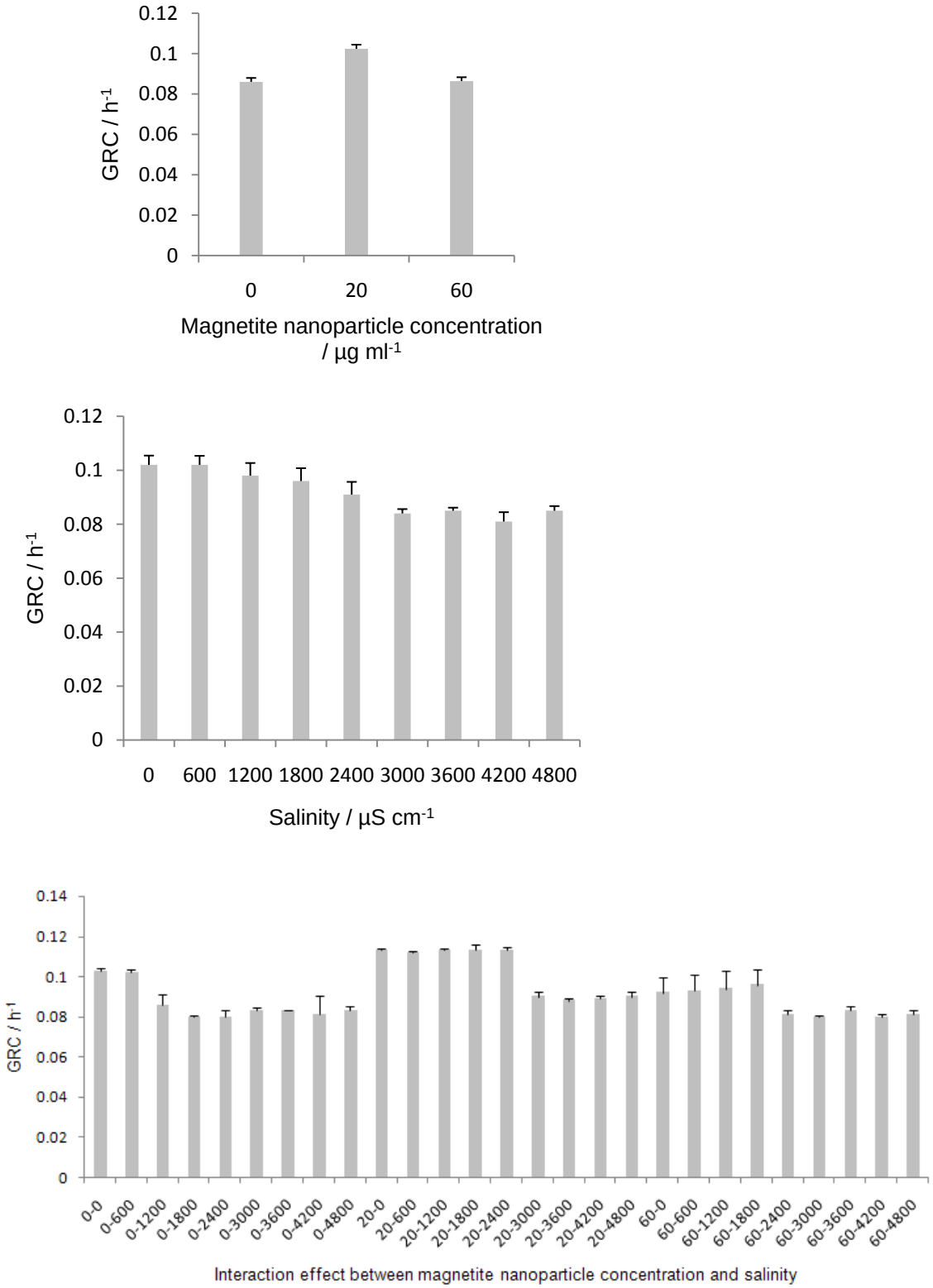

Figure 5-19. Effect of (top) magnetite nanoparticle concentration (average over all salt concentrations), (centre) salinity (average over all nanoparticle concentrations), and (bottom) their interaction (labelled as $N_{P\ conc.}$-salinity) on the growth rate constant (GRC) of the *Bradyrhizobium japonicum* Histic. Error bars show standard error.

5.5.2.4 Comparison of growth indices means (MGT, NG, and GRC) of *Bradyrhizobium* growth rate under magnetite nanoparticle concentration (0, 20, 60 µg ml^{-1}) and salinity (0, 600, 1200, ..., 4800 µS cm^{-1})

5.5.2.4.1 Comparisons of average of mean generation time (MGT)

Comparisons of the MGT averages under the nanoparticles effects showed that the lowest mean of MGT was obtained by 20 µg ml^{-1} of the nanoparticles, while the greatest of MGT was obtained by the control treatment and 60 µg ml^{-1} of the nanoparticles (Table 5-57). Comparisons of salinity effects on the MGT showed that the control treatment and 600 µS cm^{-1} of salt concentration in liquid culture media had the lowest the MGT means compared to the other concentrations (Table 5-58). The significant interaction was due to the salinity having no effect on the MGT when 20 µg ml^{-1} of the nanoparticles were present compared to the control and 80 µg ml^{-1} of the nanoparticles (Table 5-59).

Table 5-57. Comparison of the MGT means of *Bradyrhizobium japonicum* Histic under magnetite nanoparticle concentration effect.

Magnetite nanoparticle concentration / µg ml^{-1}	Mean of mean generation time (MGT) / h	LSD value = 0.2953	LSD value = 0.3916
		Alpha = 0.05	Alpha = 0.01
0	8.121	A	a
20	6.871	B	b
60	8.154	A	a

Table 5-58. Comparison of the MGT means of *Bradyrhizobium japonicum* Histic under salinity effect.

Salinity / µS cm^{-1}	Mean of mean generation time (MGT) / h	LSD value = 0.5115	LSD value = 0.6783
		Alpha = 0.05	Alpha = 0.01
0	6.871	D	c
600	6.870	D	c
1200	7.385	C	bc
1800	7.388	C	bc
2400	7.848	BC	ab
3000	8.277	AB	a
3600	8.207	AB	a
4200	8.377	A	a
4800	8.217	AB	a

Table 5-59. Comparison of the MGT means of *Bradyrhizobium japonicum* Histic under interaction effect between magnetite nanoparticle concentration and salinity.

Interaction effect between magnetite nanoparticle concentration and salinity	Mean of mean generation time (MGT) / h	LSD value = 0.8859	LSD value = 1.175
		Alpha = 0.05	Alpha = 0.01
0-0	6.74	GH	def
0-600	6.83	FGH	def
0-1200	8.44	ABCD	abc
0-1800	8.72	AB	ab
0-2400	8.64	ABC	ab
0-3000	8.33	ABCD	abc
0-3600	8.35	ABCD	abc
0-4200	8.705	AB	ab
0-4800	8.33	ABCD	abc
20-0	6.16	H	ef
20-600	6.17	H	ef
20-1200	6.11	H	f
20-1800	6.14	H	ef
20-2400	6.16	H	ef
20-3000	7.72	DE	abcd
20-3600	7.87	BCDE	abcd
20-4200	7.80	CDE	abcd
20-4800	7.71	DEF	abcd
60-0	7.72	DE	abcd
60-600	7.61	DEFG	abcd
60-1200	7.60	DEFG	bcd
60-1800	7.30	EFG	cde
80-2400	8.75	AB	ab
60-3000	8.78	A	a
60-3600	8.40	ABCD	abc
60-4200	8.63	ABC	ab
60-4800	8.61	ABC	ab

5.5.2.4.2 Comparison of number of generations means (NG)

Comparisons of the NG means under the nanoparticles effect showed that the greatest NG mean was obtained by 20 µg ml^{-1} of the nanoparticles (Table 5-60). However, comparisons of salinity conditions on the NG means showed that the greatest NG mean occurred by control treatment and 600, 1200 µS cm^{-1} of the salt concentrations (Table 5-61). The interaction effects between nanoparticle and salinity showed that combinding 20 µg ml^{-1} of the nanoparticles at the different concentrations of salt, decreased inhibitory effects of salinity conditions on the NG means (Table 5-62).

Table 5-60. Comparison of the NG means of *Bradyrhizobium japonicum* Histic under magnetite nanoparticle concentration effect.

Magnetite nanoparticle concentration / µg ml^{-1}	Mean of number of generations (NG)	LSD value = 0.6771	LSD value = 0.8980
		Alpha = 0.05	Alpha = 0.01
0	12.84	B	b
20	14.01	A	a
60	11.95	B	b

Table 5-61. Comparison of the NG means of *Bradyrhizobium japonicum* Histic under salinity effect.

Salinity / µS cm^{-1}	Mean of number of generations (NG)	LSD value =1.173	LSD value =1.555
		Alpha = 0.05	Alpha = 0.01
0	14.18	A	a
600	13.63	AB	a
1200	13.41	AB	a
1800	12.70	BC	ab
2400	12.66	BC	ab
3000	11.64	C	b
3600	11.72	C	b
4200	11.70	C	b
4800	11.08	C	b

Table 5-62. Comparison of the NG means of *Bradyrhizobium japonicum* Histic under interaction effect between magnetite nanoparticle concentration and salinity.

Interaction effect between magnetite nanoparticle concentration and salinity	Mean of number of generations (NG)	LSD value = 2.031	LSD value = 2.69a4
		Alpha = 0.05	Alpha = 0.01
0-0	14.26	AB	abcd
0-600	12.45	BCDE	def
0-1200	11.55	DE	ef
0-1800	11.01	E	f
0-2400	11.17	DE	f
0-3000	11.54	DE	ef
0-3600	11.49	DE	ef
0-4200	11.49	DE	ef
0-4800	11.59	DE	ef
20-0	15.59	A	ab
20-600	15.56	A	abc
20-1200	15.71	A	a
20-1800	15.02	A	abc
20-2400	15.60	A	ab
20-3000	12.46	BCDE	def
20-3600	12.20	CDE	def
40-4200	12.48	BCDE	def
20-4800	12.48	BCDE	def
60-0	12.68	BCDE	def
60-600	12.88	BCDE	cdef
60-1200	12.96	BCDE	bcdef
80-1800	13.06	BCD	abcdef
60-2400	11.20	DE	f
60-3000	10.94	E	f
60-3600	11.47	DE	ef
60-4200	11.13	DE	f
60-4800	11.18	DE	f

5.5.2.4.3 Comparison of means of growth rate constant (GRC)

Comparisons of the GRC means under the nanoparticles effect showed that the greatest GRC mean was obtained by 20 µg ml^{-1} of the nanoparticles, while the lowest of GRC mean was obtained by 80 µg ml^{-1} of the nanoparticles (Table 5-63). However, comparisons of salt concentrations on the GRC means showed that the greatest of GRC was obtained by control treatment and 600, 1200 and 1800 µS cm^{-1} of salt concentrations (Table 5-64). The greatest GRC means were obtained by interaction effects between 20 µg ml^{-1} of the nanoparticles and salt concentrations (Table 5-65).

Table 5-63. Comparison of the GRC means of *Bradyrhizobium japonicum* Histic under magnetite nanoparticle concentration effect.

Magnetite nanoparticle concentration / µg ml^{-1}	Mean of growth rate constant / h^{-1}	LSD value = 0.0038	LSD value = 0.0051
		Alpha = 0.05	Alpha = 0.01
0	0.086	B	b
20	0.104	A	a
60	0.0864	B	b

Table 5-64. Comparison of the GRC means of *Bradyrhizobium japonicum* Histic under salinity effect.

Salinity / µS cm^{-1}	Mean of growth rate constant / h^{-1}	LSD value = 0.0066	LSD value = 0.0088
		Alpha = 0.05	Alpha = 0.01
0	0.00102	A	a
600	0.00102	A	a
1200	0.00975	AB	ab
1800	0.00961	AB	ab
2400	0.00914	B	bc
3000	0.00841	C	cd
3600	0.00846	C	cd
4200	0.0081	C	d
4800	0.00848	C	cd

Table 5-65. Comparison of the GRC means of *Bradyrhizobium japonicum* Histic under interaction effect between nanoparticle and salinity.

Interaction effect between magnetite nanoparticle concentration and salinity	Mean of growth rate constant / h^{-1}	LSD value = 0.0114	LSD value = 0.0152
		Alpha = 0.05	Alpha = 0.01
0-0	0.103	ABC	ab
0-600	0.102	BC	ab
0-1200	0.086	DEFG	cde
0-1800	0.08	GH	de
0-2400	0.081	FGH	cde
0-3000	0.083	EFGH	cde
0-3600	0.083	EFGH	cde
0-4200	0.081	FGH	cde
0-4800	0.083	EFGH	cde
20-0	0.113	AB	a
20-600	0.112	AB	a
20-1200	0.113	A	a
20-1800	0.113	A	a
20-2400	0.113	AB	a
20-3000	0.09	DEFG	bcd
20-3600	0.089	DEFG	bcde
20-4200	0.089	DEFG	bcd
20-4800	0.09	DEFG	bcd
60-0	0.092	CDEF	bcd
80-600	0.093	CDE	bcd
60-1200	0.094	CDE	bcd
60-1800	0.096	CD	bc
60-2400	0.081	FGH	cde
60-3000	0.079	GH	de
60-3600	0.083	EFGH	cde
60-4200	0.080	FGH	de
60-4800	0.081	FGH	cde

5.5.2.5 Response of water activity (a_w) to magnetite nanoparticle concentration (0, 20, 60 µg ml^{-1}) and salinity (0, 600, 1200, ..., 4800 µS cm^{-1})

The a_w was not affected by magnetite nanoparticle concentration (Table 5-66). The only salinity and interaction effect between nanoparticle and salinity significantly influenced on the a_w in the liquid culture media at zero time and after 7days (Table 5-66). The results also showed that salinity decreased the water activity at zero time and after 7 days compared to the control treatment (Figure 5-20). However, using the nanoparticle in the different salt concentrations could prevent reduction effect of salinity on the water activity (Figure 5-20).

Table 5-66. Mean squares from analysis of variation of the water activity (a_w).

Source of Variation	df	Mean squares	
		Zero time	After 7 days
Blocks	3	0.000011	0.000081
Nanoparticles† (N_P)	2	0.000003$^{n.s.}$	0.000020$^{n.s.}$
Salinity (S)	8	0.000041**	0.000249**
N_P × S	16	0.000018**	0.000024*
Residual	78	0.000007	0.000015

† Magnetite nanoparticles (0, 20, 60 µg ml^{-1}), ** *F* test indicated significance at $P< 0.01$; $^{n.s.}$ indicates a nonsignificant result.

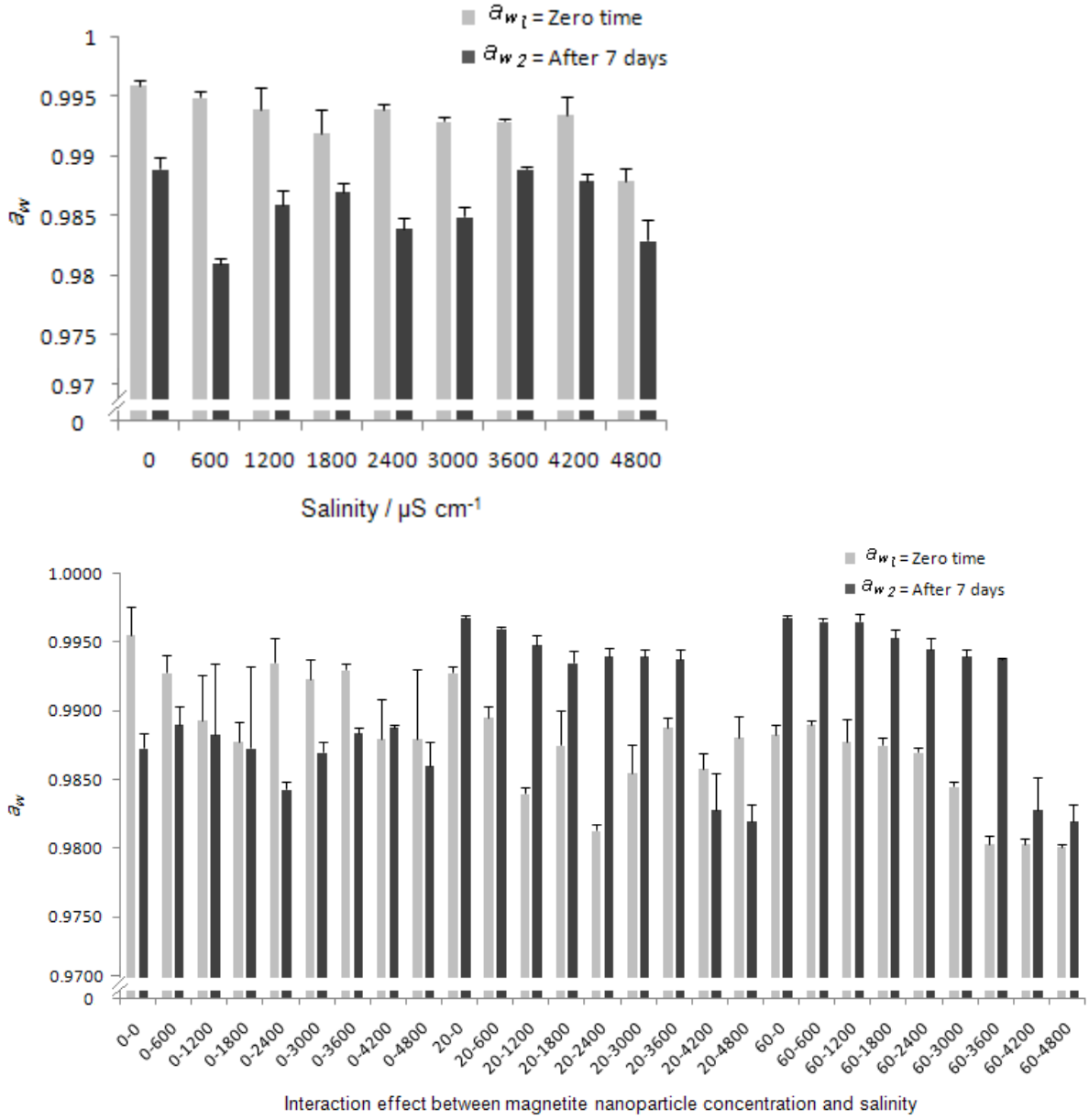

Figure 5-20. Response of water activity (a_w) to (top) salinity (average over all nanoparticle concentrations) and (bottom) interaction effect between nanoparticle and salinity (labelled as $N_{P\ conc.}$-salinity); gray bars and black bars indicate measured times at zero time and after 7 days of bacterial growth, respectively; error bars were based on standard error.

5.5.2.5.1 Comparisons of water activity (a_w) means under salinity (0, 600, 1200, ..., 4800 µS cm^{-1}) and interaction effect between salinity and the nanoparticle concentrations (0, 20, 60 µg ml^{-1})

Comparisons of the water activity means under salinity effect showed that except 600 µS cm^{-1} of salt concentration, the water activity means by the control treatment at both measured times (zero time and after 7 days) was greater than the other treatments (Table 5-67and 68). However, comparisons of the water activity means under interaction effects showed that the water activity mean was increased more by 20 µg ml^{-1} of the nanoparticles with 600 µS cm^{-1} of salt concentration, compared to the other interaction effects after 7 days (Table 5-69 and 70).

Table 5-67. Comparison of water activity means at zero time under salinity effect in liquid culture media of *Bradyrhizobium japonicum* Histic.

Salinity / µS cm^{-1}	Mean of water activity (a_w) at zero time	LSD value = 0.00217	LSD value = 0.0029
		Alpha = 0.05	Alpha = 0.01
0	0.9963	A	a
600	0.9951	AB	ab
1200	0.9935	BC	abc
1800	0.9922	C	c
2400	0.9940	BC	abc
3000	0.9934	BC	bc
3600	0.9935	BC	abc
4200	0.9883	D	d
4800	0.9883	D	d

Table 5-68. Comparison of water activity means after 7 days under salinity effect in liquid culture media of *Bradyrhizobium japonicum* Histic.

Salinity / µS cm^{-1}	Mean of water activity (a_w) after 7 days	LSD value = 0.0032	LSD value = 0.0042
		Alpha = 0.05	Alpha = 0.01
0	0.9894	A	a
600	0.9892	A	a
1200	0.9867	ABC	ab
1800	0.9874	AB	ab
2400	0.9842	CD	b
3000	0.9857	BCD	ab
3600	0.9857	BCD	ab
4200	0.9848	BCD	b
4800	0.9833	D	b

Table 5-69. Comparison of water activity means at zero time under interaction effect between magnetite nanoparticle concentration and salinity in liquid culture media of *Bradyrhizobium japonicum* Histic.

Interaction effect between magnetite nanoparticle concentration and salinity	Mean of water activity (a_w) at zero time	LSD value = 0.0038	LSD value = 0.0050
		Alpha = 0.05	Alpha = 0.01
0-0	0.9955	A	a
0-600	0.9928	AB	abcd
0-1200	0.9893	BCD	bcdefg
0-1800	0.9878	DEF	efgh
0-2400	0.9935	A	ab
0-3000	0.9923	ABC	abcde
0-3600	0.9930	AB	abc
0-4200	0.9880	DEF	defgh
0-4800	0.9880	DEF	defgh
20-0	0.9928	AB	abcd
20-600	0.9895	BCD	bcdef
20-1200	0.9840	GHI	hij
20-1800	0.9875	DEFG	efgh
20-2400	0.9813	HIJ	ij
20-3000	0.9855	EFG	fghi
20-3600	0.9888	CDE	bcdefgh
20-4200	0.9858	DEFG	fghi
20-4800	0.9881	DEF	cdefgh
60-0	0.9883	DE	cdefgh
60-600	0.9890	CDE	bcdefg
60-1200	0.9878	DEF	efgh
60-1800	0.9875	DEFG	efgh
80-2400	0.9870	DEFG	fgh
60-3000	0.9845	FGH	ghij
60-3600	0.9803	IJ	j
60-4200	0.9803	IJ	j
60-4800	0.9801	J	j

Table 5-70. Comparison of water activity means after 7 days under interaction effect between magnetite nanoparticle concentration and salinity in liquid culture media of *Bradyrhizobium japonicum* Histic.

Interaction effect between magnetite nanoparticle concentration and salinity	Mean of water activity (a_w) after 7 days	LSD value = 0.0055	LSD value = 0.0073
		Alpha = 0.05	Alpha = 0.01
0-0	0.9873	EFG	def
0-600	0.9890	BCDE	bcdef
0-1200	0.9883	DEF	cdef
0-1800	0.9873	EFG	def
0-2400	0.9843	EFG	f
0-3000	0.9870	EFG	ef
0-3600	0.9884	DE	cdef
0-4200	0.9888	CDE	bcdef
0-4800	0.9860	EFG	f
20-0	0.9968	A	a
20-600	0.9960	A	ab
20-1200	0.9948	A	abc
20-1800	0.9935	ABCD	abcde
20-2400	0.9940	ABC	abcde
20-3000	0.9940	ABC	abcde
20-3600	0.9938	ABCD	abcde
20-4200	0.9828	FG	f
20-4800	0.9820	G	f
60-0	0.9968	A	a
80-600	0.9965	A	a
60-1200	0.9965	A	a
60-1800	0.9953	A	abc
60-2400	0.9945	AB	abcd
60-3000	0.9940	ABC	abcde
60-3600	0.9938	ABCD	abcde
60-4200	0.9828	FG	f
60-4800	0.9820	G	f

5.5.2.6 Response of oxidation reaction potential (ORP) to magnetite nanoparticle concentration (0, 20, 60 µg ml^{-1}) and salinity (0, 600, 1200, ..., 4800 µS cm^{-1})

The ORP was affected by nanoparticles and interaction effect between nanoparticle and salinity (Table 5-71). The results showed that main effect of salinity had no significant influence on the ORP (Table 5-71). However, after 7 days, by 20 µg ml^{-1} of the nanoparticles, reduction of the ORP was greater than other treatments (Figure 5-21)

Table 5-71. Mean squares from analysis of variation of the oxidation reaction potential (ORP).

Source of Variation	df	Mean squares	
		Zero time	After 7 days
Blocks	3	193.5	631.3
Nanoparticles† (N_P)	2	722**	3804**
Salinity (S)	8	80.1$^{n.s.}$	127.4$^{n.s.}$
N_P × S	16	38.3$^{n.s.}$	323.1**
Residual	78	80.6	152.3

† Magnetite nanoparticles (0, 20, 60 µg ml^{-1}); ** *F* test indicated significance at $P< 0.01$; $^{n.s.}$ indicates a nonsignificant result.

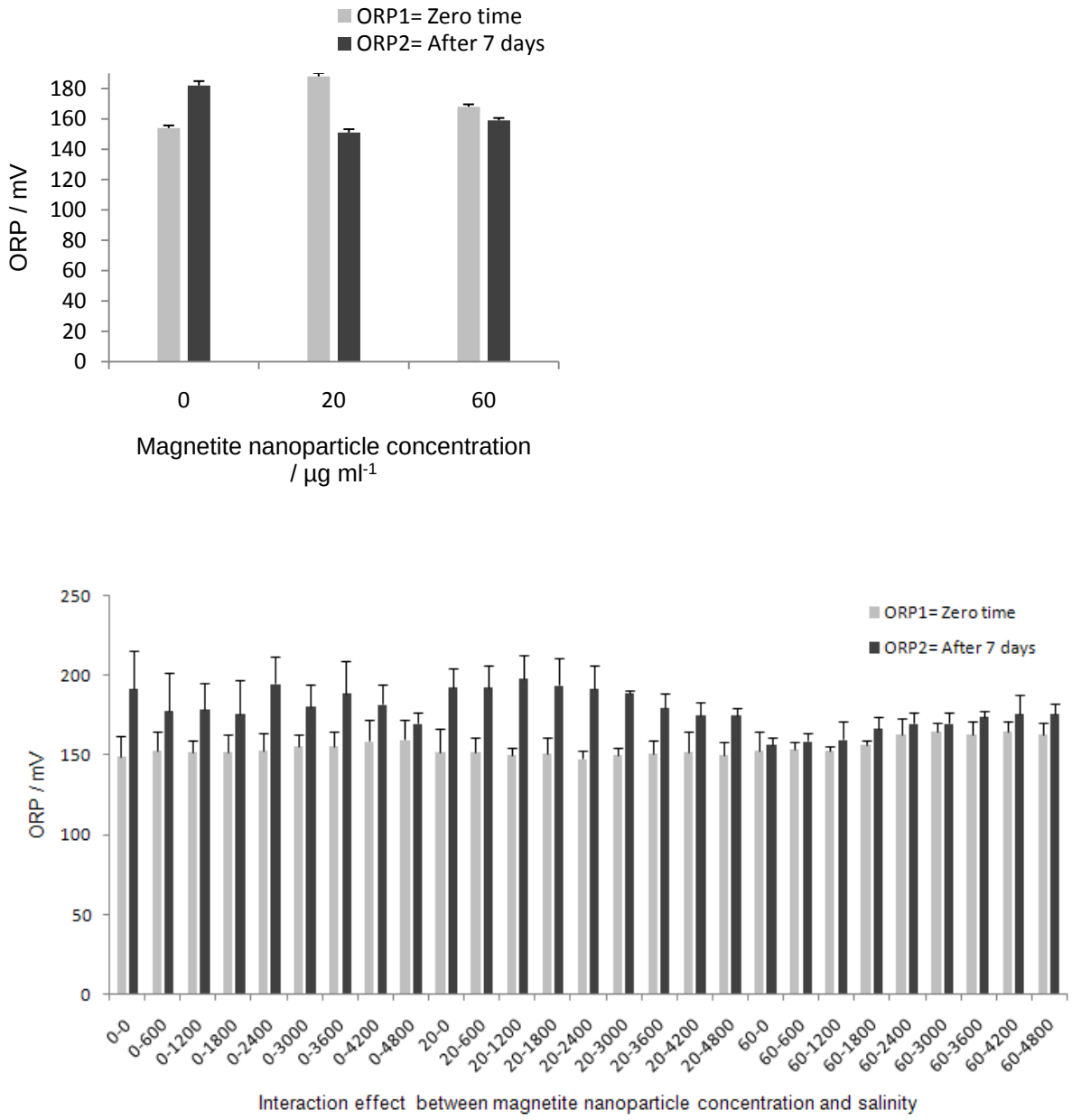

Figure 5-21. Response of the ORP to (top) the nanoparticles (average over all salt concentrations) and (bottom) interaction effect between nanoparticle and salinity (labelled as $N_{P\ conc.}$-salinity); gray bars and black bars indicate measured times at zero time and after 7 days of bacterial growth, respectively. Error bars show standard error.

5.5.2.6.1 Comparison of oxidation reaction potential (ORP) means under magnetite nanoparticle concentration (0, 20, 60 µg ml^{-1}) and interaction effect between nanoparticles and salinity (0, 600, 1200, ..., 4800 µS cm^{-1})

Comparisons of the ORP means under the nanoparticles showed that the greatest of ORP mean occurred by 60 µg ml^{-1} of the nanoparticles at zero time, in contrast after 7 days, this concentration caused to occur the lowest of ORP mean compare to the other treatments (Table 5-72 and 73). Comparisons of the ORP means under interaction effect between the nanoparticles and salinity showed that at zero time were not significant (Table 5-71). However, after 7 days, the ORP means were decreased by 60 µg ml^{-1} of the nanoparticles with 600 to 1800 µS cm^{-1} salt concentrations means compared to the other treatments (Tables 5-74).

Table 5-72. Comparison of means of magnetite nanoparticle concentration effect on oxidation reaction potential (ORP) at zero time of *Bradyrhizobium japonicum* Histic.

Magnetite nanoparticle concentration / µg ml^{-1}	Mean of oxidation reaction potential (ORP) at zero time	LSD value = 4.213	LSD value = 5.587
		Alpha = 0.05	Alpha = 0.01
0	154.3	A	a
20	187.5	B	b
60	167.7	C	c

Table 5-73. Comparison of means of magnetite nanoparticle concentration effect on oxidation reaction potential (ORP) after 7 days of *Bradyrhizobium japonicum* Histic

Magnetite nanoparticle concentration / µg ml^{-1}	Mean of oxidation reaction potential (ORP) after 7 days	LSD value = 5.791	LSD value = 7.680
		Alpha = 0.05	Alpha = 0.01
0	182.4	A	a
20	150.5	B	b
60	159.4	B	b

Table 5-74. Comparison of means of interaction effect between magnetite nanoparticle concentration and salinity on oxidation reaction potential (ORP) after 7 days of *Bradyrhizobium japonicum* Histic.

Interaction effect between magnetite nanoparticle concentration and salinity	Mean of oxidation reaction potential (ORP) after 7 days	LSD value = 17.37	LSD value = 23.04
		Alpha = 0.05	Alpha = 0.01
0-0	192.3	ABCD	abc
0-600	177.8	BCDEF	abcdef
0-1200	179.3	BCDEF	abcdef
0-1800	176.3	CDEFG	abcdef
0-2400	194.8	AB	ab
0-3000	180.5	ABCDEF	abcde
0-3600	188.5	ABCDE	abcd
0-4200	182.3	ABCDEF	abcde
0-4800	170.5	FGH	cdef
20-0	192.8	ABC	abc
20-600	192.8	ABC	abc
20-1200	197.5	A	a
20-1800	194.0	AB	ab
20-2400	192.3	ABCD	abc
20-3000	188.5	ABCDE	abcd
20-3600	179.8	BCDEF	abcde
40-4200	174.8	EFG	abcdef
20-4800	175.0	DEFG	Abcdef
60-0	156.5	H	f
60-600	159.3	GH	ef
60-1200	160.3	GH	ef
80-1800	166.8	FGH	def
60-2400	170.3	FGH	cdef
60-3000	170.0	FGH	cdef
60-3600	174.3	EFG	bcdef
60-4200	175.8	CDEFG	abcdef
60-4800	176.3	CDEFG	abcdef

5.5.3 Response of the number of viable cells to salinity and magnetite nanoparticle effects

The results showed that the average of the maximum number of *Bradyrhizobium* cells was affected under salt concentrations (Figure 5-22). The average of the maximum number of the viable cells obtained after 96 h from 600 to 1800 $\mu S\ cm^{-1}$ of salt concentrations (Figure 5-22). However, increasing concentration of salinity in liquid culture media, caused to obtain the average of the maximum number of viable cells after 72 h, and increase in salinity concentration was probably as an inducer factor in the bacteria growth. In addition, reduction of the number of viable cells in the higher salinity concentration was greater than the lower salinity concentration (Figure 5-22).

Effect of magnetite nanoparticle concentration on the cell viability showed that the highest average of the maximum number of viable *Bradyrhizobium* cells (about 7×10^8 viable cells per ml) obtained in the 20, 40 and 60 $\mu g\ ml^{-1}$ compared to the other treatments(Figure 5-23).However, after 96 h, the average of the maximum number of viable cells only obtained by 40 $\mu g\ ml^{-1}$ of the nanoparticles, and also it had the lowest reduction of cell viability compared to the other concentrations (Figure 5-23).

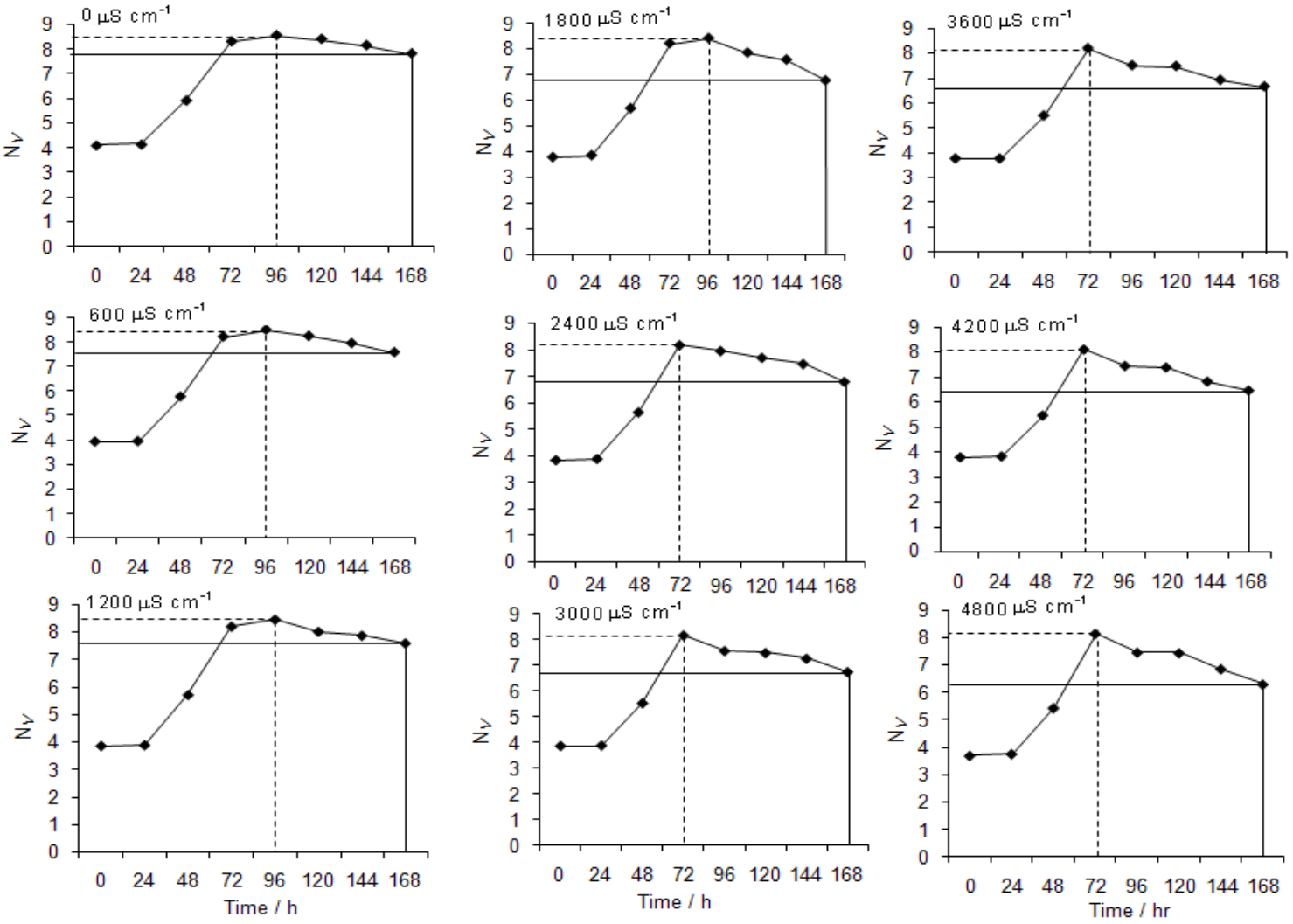

Figure 5-22. Effect of salinity (average over all nanoparticle concentrations) on average number of viable of *Bradyrhizobium* cells in liquid cultur media.

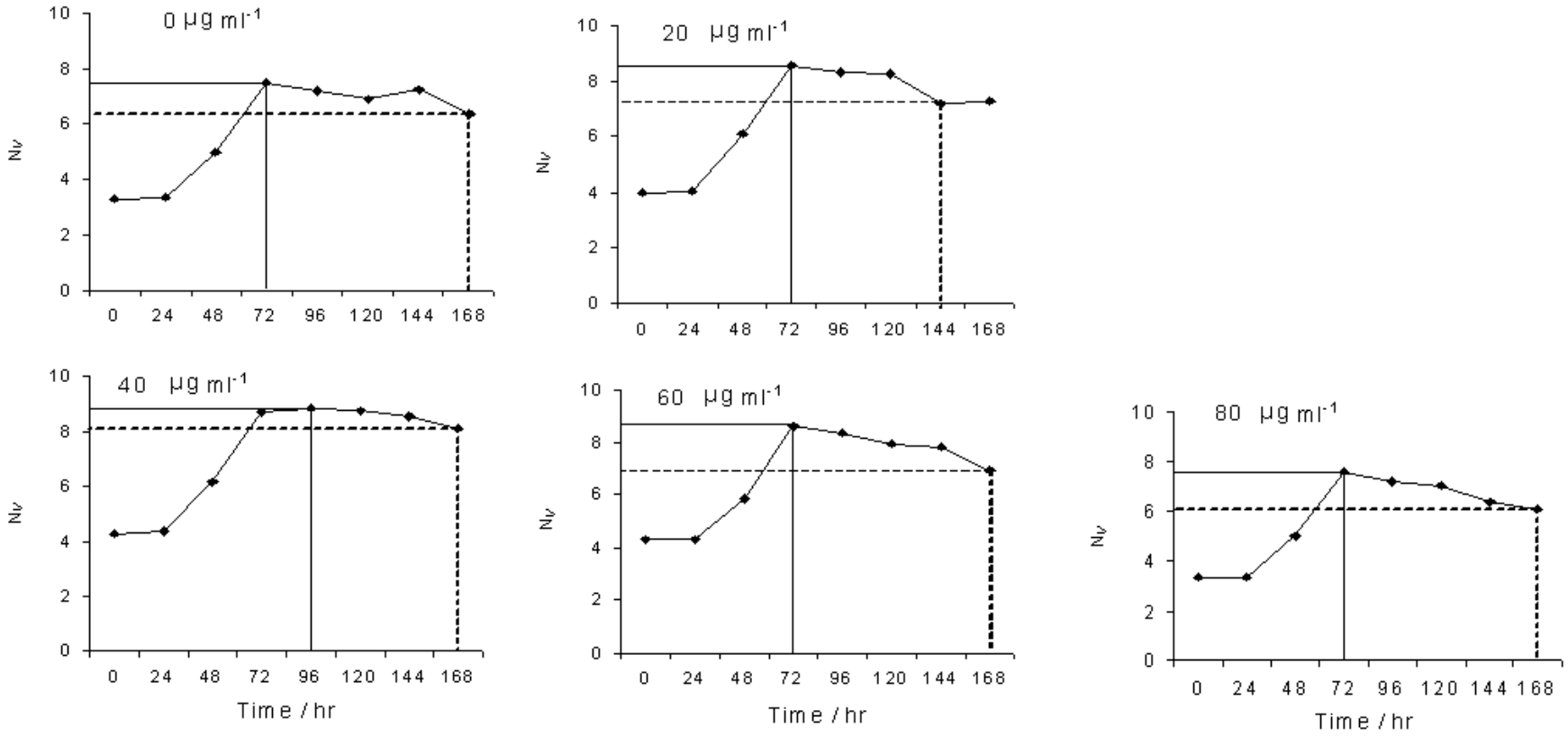

Figure 5-23. Effect of magnetite nanoparticle concentration (average over all salt concentrations) on the average maximum number of viable *Bradyrhizobium* cells in liquid culture media.

5.5.4 Discussion

The literature suggested that among of *Bradyrhizobium* strains, *B. japonicum* is salt-sensitive [229]. The results indicated that the growth and viability of cells decreasd due to salinity stress. Negative response of the growth indices (MGT, NG and GRC) of *Bradyrhizobium* cells to salinity showed that increasing salinity concentration probably was due to increasing density of reactive radical molecules in the niche of the bacterial growth, since the ORP increased, and in contrast the water activity decreased. However, using 20 and 40 μg ml^{-1} of the nanoparticles approximately could reduce negative effects of salinity on the bacterial growth. The literature suggested one of the most negative impacts of salinity stress, is iron deficiency, thus likely the nanoparticles could compensate the iron deficiency for the bacteria, when the bacteria experienced salinity stress. From control treatment to 1800 μS cm^{-1}, the average of the maximum number of viable cells obtained after 96 h was about 7×10^8 viable cells of bacteria, whereas at higher salt concentrations (from 1800 to 4800 μS cm^{-1}), the maximum number of viable cells decreased to about 10^7 viable cells, and also the maximum number of viable cells was obtained after only 72 h. Although salinity stress was able to induce earlier occurrence of the maximum population, it also led to more rapid death of the bacterial cells. In fact increasing concentration of sodium ions caused damage to the cell wall, decreasing water availability, and also leading to an increased quantity of radical molecules in the culture medium. These responses were consistent with previous researches; Lioret *et al*. (1998) and Ruberg *et al*. (2003) showed that salt stress induce *rhizobia* to change cell morphology, and the dimension and modification of their polysaccharides very important in the symbiotic mechanism, like exopolysaccharides (EPS) and lipopolysaccharides (LPS) [145,219].

Furthermore, previous studies illustrated that under salinity stress and growth-limiting stresses cells of *rhizobia* accumulate compatible solutes (and prefer their uptake over synthesis within the cytoplasm) such as: K^+ ions, glycogen, sucrose, trehalose, maltose, cellobiose, turanose, gentiobiose, palatinose and amino acids like glutamate and proline, which are osmoprotective for several solutes, and carbohydrates and disaccharides. In fact, accumulation of osmoprotectants within the

cell is often greater than that of the environment and provides the cell with sufficient turgor pressure to prevent cell lysis [18,48,258].

Based on the results obtained the response of physiological indices of bacterial growth was inferred to be affected by salinity stress. The number of generations (NG) decreased from 14 to 11 and/or the MGT increased from 6.5 to 8.5 h. However, the results indicated that the bacterial cells could withstand high concentrations of salinity, so that from 1800 to 4800 µS cm^{-1} the responses of physiological indices were similar (Figures 5-19, 20, and 21). Nanoparticles residing on the cells wall and complexing of the nanoparticles and the EPS and the LPS could preserve the cell wall. Vriezen *et al.* (2007) showed that response to salinity stress vary highly between *Rhizobium* strains [258]. They suggested a general response model of *rhizobia* to environmental salinization, based on declining general metabolism after an osmotic upshift. Likewise, Domingguez-Ferreas *et al.* (2006) reported that osmotic stress led to induction of a large number of genes having unclear functions and inhibition of many genes coding for proteins with known functions [50]. For example one-fourth of all gene productions specifically downregulated by salt (NaCl) stress encoded ribosomal proteins.

However, in the presence of the nanoparticles bacterial cells could withstand salinity stress. The nanoparticles, via redox potential and buffering effect, could diminish direct/indirect negative effects of the sodium ions on water availability, the requirement for oxygen for respiration of the bacterial cells and metabolic activities in the interior of bacterial cells, and reactive molecules in culture medium. Oxygen deficiency for bacteria growth is initially deletrious under salinity stress [46].

In order to take up iron, some investigations have shown that, bacteria use the complex compounds, which are composed of ferric-specific ligands (siderophores) and their cognate membrane receptors [92,93]. Bacteria generally can accumulate iron both with self-made siderophores and with siderophores that they themselves do not synthesize [93], hence, exudation of these compounds can cause uptake of the nanoparticles. Physical and chemical properties of the nanoparticle such as size, shape and redox potential could accelerate up take of the nanoparticles by bacteria. Thus this mechanism could be one of the strategies for enhancing cell viability under extreme conditions [153]. Furthermore, dissimilatory iron or iron respiration uses

ferric ion as a terminal electron acceptor for the purpose of energy generation during anaerobic respiration (when bacteria experience adverse environmental conditions). The released siderophores complex ferric ions in the environment exterior to the bacterial cell, then these ferric-specific ligands deliver the ferric ions to the cell, and after reducing to the ferrous from is taken up [153, 92,93]

Thus, in order to compensate for oxygen deficiency by extreme conditions, the bacteria induce and use the iron respiration mechanism [153], hence the nanoparticles (if taken up) may be involved in promotion of iron respiration and compensate for oxygen deficiency for allowing respiration of the bacteria under salinity stress. In addition these nanoparticles could scavenge radical molecules discharged from the bacteria. The results indicated that the ORP in the presence of the nanoparticles decreased, and/or the a_w increased, because of which the death of *Bradyrhizobium* cells was considerably decreased and they could even withstand high concentrations of salt. In addition the nanoparticles could act possibility as osmoprotectatnts.

Moreover, application of high concentration of the nanoparticles may induce osmotic pressure in the medium, and also induce releasing of ferric uptake repressor (Fur) proteins of the bacterial cells [27]. These proteins when are released that density of exterior iron particles abound, as there particles can limit growth activity of bacteria via oxidizing solutes and reduction pH, hence at higher concentrations, the nanoparticles imply a inhibitor effect on the bacterial growth [27].

5.5.5 Conclusions

According to the results, magnetite nanoparticles could diminish the effects of salinity stress on the BGR. 20 and 40 μg ml^{-1} of the nanoparticles could increase resistance of the bacterial cells under salinity stress.

Properties of the nanoparticles such as their redox potential, buffering effect, ability to scavenge radical molecules, involvement in respiration of the bacterial cells, and acting as osmoprotectants could decrease the effects of salinity stress. Nanoparticles residing the on the cell wall presumably prevent damage to the cell wall and water and oxygen deficiency.

However the degree of benefit of the nanoparticles depends on their concentration, and high concentrations inversely affect the response to salinity stress.

5.6 Effects of desiccation, temperature, seed and magnetite nanoparticles on *Bradyrhizobium japonicum* viability as inocula

The results showed that the cell viability was affected by desiccation, temperature, nanoparticles, interaction between desiccation and temperature, interaction between desiccation and nanoparticle, interaction between temperature and SG, interaction between temperature and nanoparticle and interaction between SG and nanoparticle (Table 5-75) However the other effects, although they changed the cell viability mean, were not significant.

Table 5-75. Mean squares from analysis of variation of the cell viability (N_l).

Source of Variation	df	Mean squares of the N_l
Blocks	3	4.33
Desiccation (D)	5	343.53**
Temperature (Temp.)	1	30.38**
Seed / Glass beads (SG)	1	$2.01^{n.s.}$
Nanoparticles (N_P)	5	20.34**
D × Temp.	5	5.36*
D × SG	5	$1.02^{n.s.}$
D × N_P	25	4.03*
Temp. × SG	1	12.65*
Temp. × N_P	5	5.36*
SG × N_P	5	6.10*
D × Temp. × SG	5	$1.70^{n.s.}$
D × T × N_P	25	$2.39^{n.s.}$
D × SG × N_P	25	$1.46^{n.s.}$
Temp. × SG × N_P	5	$2.27^{n.s.}$
D × Temp. × SG × N_P	25	$3.19^{n.s.}$
Residual	429	2.36

* and ** *F* test indicates significance at $P < 0.05$ and $P < 0.01$, respectively; $^{n.s.}$ indicates a nonsignificant result.

5.6.1 Effect of desiccation on the viability

Effect of desiccation times significantly decreased the viability. However, the results also showed that more than 50 % of the inocula on SG were survived after 96 h, (Figure 5-24 and Table 5-76). The significant interaction effect between desiccation and temperature showed that from zero time to 96 h of the desiccation times in presence of the 4 ºC, the viability means were greater than 25 ºC (Table 5-77). Comparisons of the viability means under the significant interaction effect between desiccation and nanoparticles showed that the viability means in presence of the nanoparticles were greater than the control treatment, and also after 96 h, the highest viability was obtained by 60 and 40 µg $ml^{-1}$of the nanoparticles (Table 5-78).

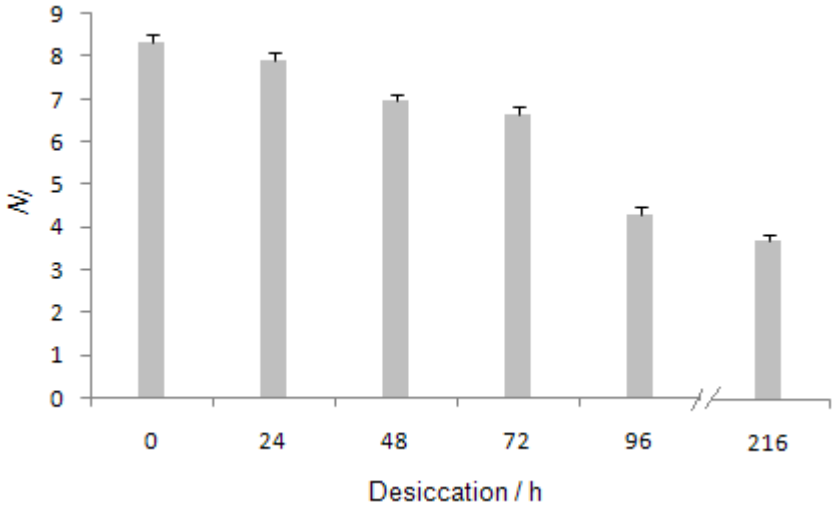

Figure 5-24. Response of cell viability to the desiccation. Error bars show standard error.

Table 5-76. Comparison of the viability mean (N_i).under desiccation effect.

Desiccaion / h	Mean of the viability	LSD value = 0.4359 Alpha = 0.05	LSD value = 0.5738 Alpha = 0.01
0	8.331	A	a
24	7.914	A	a
48	6.989	B	b
72	6.636	B	b
96	4.291	C	c
216	3.742	D	c

Table 5-77. Comparisons of the viability mean under interaction effect between desiccation and temperature effect.

Interaction effect between desiccation and temperature	Mean of the viability	LSD value = 0.6165 Alpha = 0.05
0-4 °C	8.3853	A
24-4 °C	8.0661	ABC
48-4 °C	7.6443	C
72-4 °C	6.8783	D
96-4 °C	4.5749	E
216-4 °C	3.7310	F
0-25 °C	8.2758	AB
24-25 °C	7.7611	BC
48-25 °C	6.3336	D
72-25 °C	6.3935	D
96-25 °C	4.0062	EF
216-25 °C	3.7538	F

Table 5-78. Comparisons of the viability means under interaction effect between desiccation and nanoparticles.

Interaction effect between magnetite nanoparticle concentration and desiccation	Mean of the viability	LSD value = 1.007
		Alpha = 0.05
0-0	8.669	A
0-24	7.386	BCD
0-48	6.258	EF
0-72	5.655	FG
0-96	3.501	I
0-216	3.624	I
20-0	8.680	A
20-24	8.079	AB
20-48	7.552	BCD
20-72	7.981	ABC
20-96	4.366	HI
20-216	3.869	I
40-0	8.195	AB
40-24	8.671	A
40-48	7.613	BCD
40-72	8.007	ABC
40-96	5.001	GH
40-216	3.886	I
60-0	8.165	AB
60-24	8.065	AB
60-48	6.874	DE
60-72	7.057	CDE
60-96	5.084	HG
60-216	3.720	I
80-0	8.696	A
80-24	7.705	ABCD
80-48	6.826	DE
80-72	5.503	FG
80-96	3.881	I
80-216	3.711	I
100-0	7.579	BCD
100-24	7.576	BCD
100-48	6.810	DE
100-72	5.612	FG
100-96	3.911	I
100-216	3.645	I

5.6.2 Effect of temperature on the viability

The results of temperature effect showed that at 4 ºC, the viability means were greater than 25 ºC (6.55 and 6.09 log number of viable cell, respectively) (Figure 5-25). The significant interaction effects between the temperature and the SG showed that the glass beads at 25 ºC had the lowest viability compared to the other treatments (Table 5-79). The results also showed that both temperatures (4 and 25 ºC) in presence of the nanoparticles increased the viability compared to the control treatment, however, the highest viability was obtained by 40 µg ml^{-1} of the nanoparticles at 4 ºC (Table 5-80)

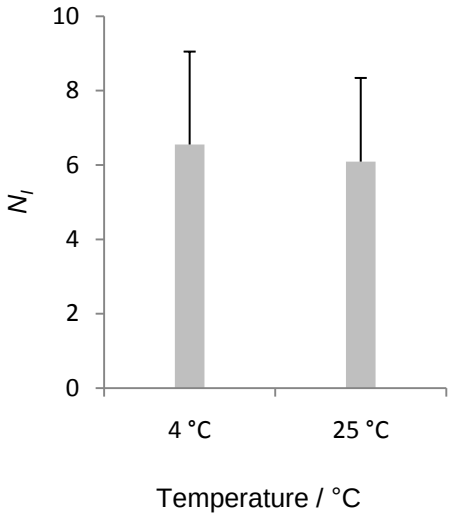

Figure 5-25. Effect of temperature on the viability of *Bradyrhizobium japonicum*. Error bars show standard error.

Table 5-79. Comparisons of the viability means under interaction effect between temperature and the seeds and glass beads (SG).

Interaction effect between temperature and SG	Mean of the viability	LSD value = 0.3559 Alpha = 0.05
4°C - Glass beads	6.754	B
4°C - Seed	6.339	B
25°C - Glass beads	5.998	A
25°C - Seed	6.179	B

Table 5-80. Comparisons of the viability means under interaction effect between temperature and the nanoparticles.

Interaction effect between magnetite nanoparticle concentration and temperature	Mean of number of viable cells	LSD value = 0.6165 Alpha = 0.05
0-4 °C	6.507	ABC
20-4 °C	6.746	AB
40-4 °C	7.022	A
60-4 °C	6.589	ABC
80-4 °C	6.375	BC
100-4 °C	6.042	CD
0-25 °C	5.191	E
20-25 °C	6.763	AB
40-25 °C	6.769	AB
60-25 °C	6.400	BC
80-25 °C	5.732	DE
100-25 °C	5.669	DE

5.6.3 Effect of the nanoparticles on the viability

The results showed that by 20, 40 and 60 µg ml^{-1} of the nanoparticles, the viability increased compared to the other treatments (Figure 5-26 and Table 5-81). The significant interaction effect between the nanoparticles and the SG showed that in the presence 20 and 40 µg ml^{-1} of the nanoparticles, the viability means on the seeds were greater than other treatments (Table 5-82). In contrast, at higher concentrations of the nanoparticles, the viability means on glass beads were greater than other treatments (Table 5-82).

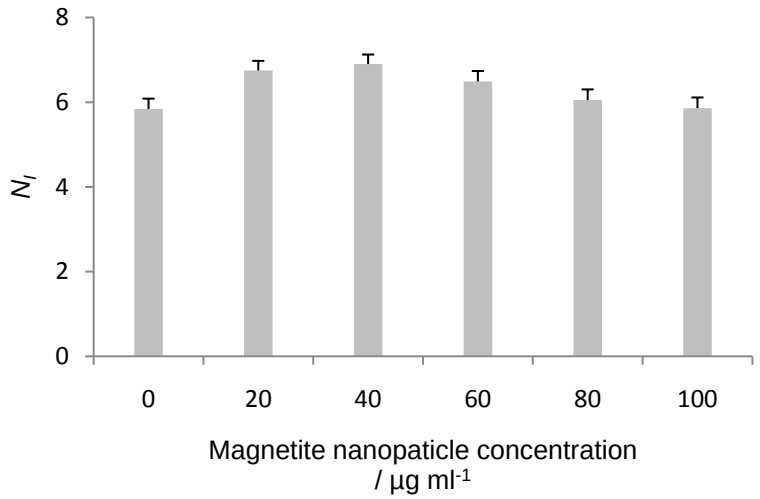

Figure 5-26. Effect of nanoparticles on the viability of *Bradyrhizobium japonicum*. Error bars show standard error.

Table 5-81. Comparisons of the viability means under magnetite nanoparticle concentration effect.

Magnetite nanoparticle concentration / µg ml^{-1}	Mean of number of viable cells	LSD value = 0.4359	LSD value = 0.5738
		Alpha = 0.05	Alpha = 0.01
0	5.849	B	c
20	6.754	A	a
40	6.896	A	a
60	6.494	A	ab
80	6.054	B	bc
100	5.855	B	c

Table 5-82. Comaprisons of the viability means under interaction effect between the nanoparticles and the SG.

Interaction effect between glass beads and seed and magnetite nanoparticle	Mean of number of viable cells	LSD value = 0.06157
		Alpha = 0.05
Glass beads-0	5.691	H
Glass beads-20	6.668	C
Glass beads-40	6.785	B
Glass beads-60	7.020	A
Glass beads-80	6.170	D
Glass beads-100	5.923	F
Seed-0	6.007	E
Seed-20	6.840	B
Seed-40	7.006	A
Seed-60	5.969	EF
Seed-80	5.937	F
Seed-100	5.787	G

5.6.4 Discussion

According to the results and their analysis several factors affecting cells viability of *Bradyrhizobium* on seeds were recognized. The following possible explanations seem worth considering:

1. The results of desiccation effects on inocula viability indicate that at low temperature (4 °C) the negative effect of desiccation on the viability decreased. Using the nanoparticles might promote hygroscopicity, oxygen and carbon in the medium. This result is consistent with the fact that the positive effect of salt or other osmotic materials in the medium on the accumulation of some secreted substances such as osmotic regulators (e.g., some strains of *Bradyrhizobium* enhance their capacity to oxidize carbon sources by increasing growth rate and exopolysaccharide production involved in adhesion, resulting in a greater adaptive capacity to colonize unfavourable saline environments) [12,22,80,86], which can compensate source deficit of carbon for the growth of these bacteria.

2. The literature suggests that the nature of the suspending medium under the recovery of bacteria cells after dehydration is important for the survival of the inocula in the dry state [44,258], and also suggests that the moisture stress response of *rhizobia* strains is morphological change [29,278]. Releasing certain compounds (e.g., polysaccharide, disaccharides like trehalose) improves the ability of inocula to persist under extreme conditions [28,44,55,258]. Adding nanoparticles into the culture medium, was probably able to enhance the release of compounds like polysaccharide and disaccharide which can absorb more water, hence increase the viability: preserving the moist layer on seeds is important for survival of bacteria [235,236]. The results of the interaction effects analysis revealed some factors whereby nanoparticles could increase the viability of cells, but there is no direct comparison of these results to the literature, and also it is difficult because of diverse experimental methods [39,155,169].

3. From the low temperature results are may infer that the nanoparticles control dehydration and thereby could achieve preservation of cell viability on seeds.

This inference is consistent with the fact that the low temperature can enhance cell viability [17,47,134,166].

5.6.5 Conclusions

Inocula viability of *Bradyrhizobium japonicum* is affected by magnetite nanoparticles. 20, 40 and 60 µg ml^{-1} concentrations of the nanoparticles could increase inocula viability on seeds for several desiccation times and low temperature, but use of concentrations 80 and 100 µg ml^{-1} gave almost the same results as the control treatments.

5.7 Effects of magnetite nanoparticle concentration on secreted signal molecules from soybean root and *Bradyrhizobium*, acetylene reduction and total dry matter (vegetative components) of soybean plant

5.7.1 Effect of magnetite nanoparticle concentration on genistein secreted from soybean root

According to the analysis of variation (Table 5-83) genistein secretion from the soybean root was significantly affected by *Bradyrhizobium* cells coated with the nanoparticle at $P < 0.01$. The results showed that the genistein secreted means increased by using 40 and 60 µg ml^{-1} of the nanoparticle (Figure 5-27).

Table 5-83. Mean squares from analysis of variation of the genistein secreted mean from soybean root under different nanoparticle concentrations.

Source of variation	df	Mean squares of secreted genistein / µg $root^{-1}$
Blocks	2	0.009
Nanoparticles	4	0.546**
Residual	8	0.025

** *F* test indicates significance at $P < 0.01$.

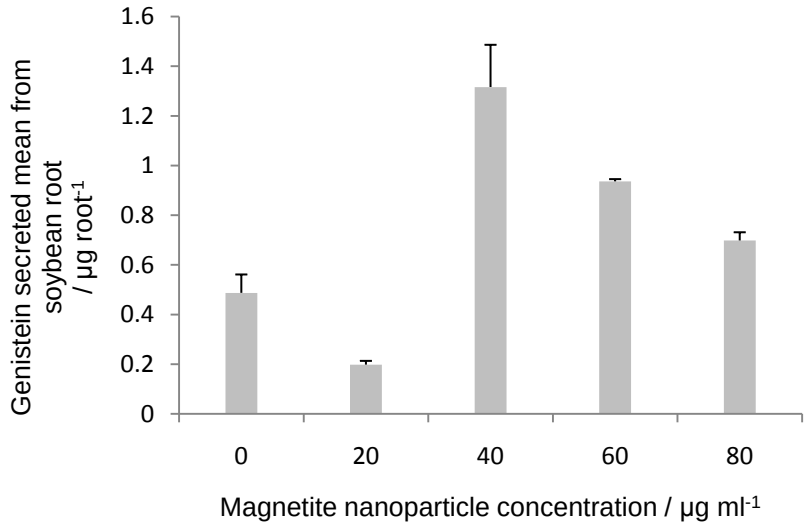

Figure 5-27. Genistein secreted means from soybean root at different concentrations of the nanoparticle. Error bars show standard error.

Comparisons of the genistein secreted means showed that the greatest mean of genistein secreted from the root was obtained by 40 µg ml^{-1} of the nanoparticles Table 5-84). However, the differences of generated genisten means, between control treatment and 20 µg ml^{-1} of the nanoparticles, were not significant, and also, between 60 and 80 µg ml^{-1} of the nanoparticles, difference of the means were not significant (Table 5-84)

Table 5-84. Comparisons of the secreted genistein means from soybean root under magnetite nanoparticle concentration effect.

Magnetite nanoparticle concentration / µg ml^{-1}	Mean of genistein secreted µg $root^{-1}$	LSD value = 0.2977	LSD value = 0.4332
		Alpha = 0.05	Alpha = 0.01
0	0.4874	CD	cd
20	0.1980	D	d
40	1.316	A	a
60	0.9362	B	ab
80	0.6980	BC	bc

5.7.2 Effect of magnetite nanoparticle concentration on excretion of lipochitooligosaccharide (LCO) from *Bradyrhizobium japonicum* Histic

Figure 5-28 shows secreted LCO at the different nanoparticle concentrations. 40 and 60 µg l^{-1} of nanoparticles had considerable affect on the secreted LCO from *Bradyrhizobium*. The results were in accordance with the retention time reported for secreted LCO by Taurian *et al*. (2008) and Soulemanov *et al*. (2002) [165,196]. Although other studies reported that the retention time (RT) observed at 30:50 until 31:00 minutes. Generally the RT depends on column type, gradient, length of tubes in the chromatograph temperature, method, and different HPLC instruments.

Moreover the nanoparticles could intensify excretion the LCO form bacterial cell in the presence of genistein. There are three possibilities for increasing secretion of the LCOs in presence of the nanoparticles: i) via increasing number of viable cells, ii) by increasing amount of LCO secreted by bacterial cell, and iii) simultaneously increasing survival and amount of secreted LCOs.

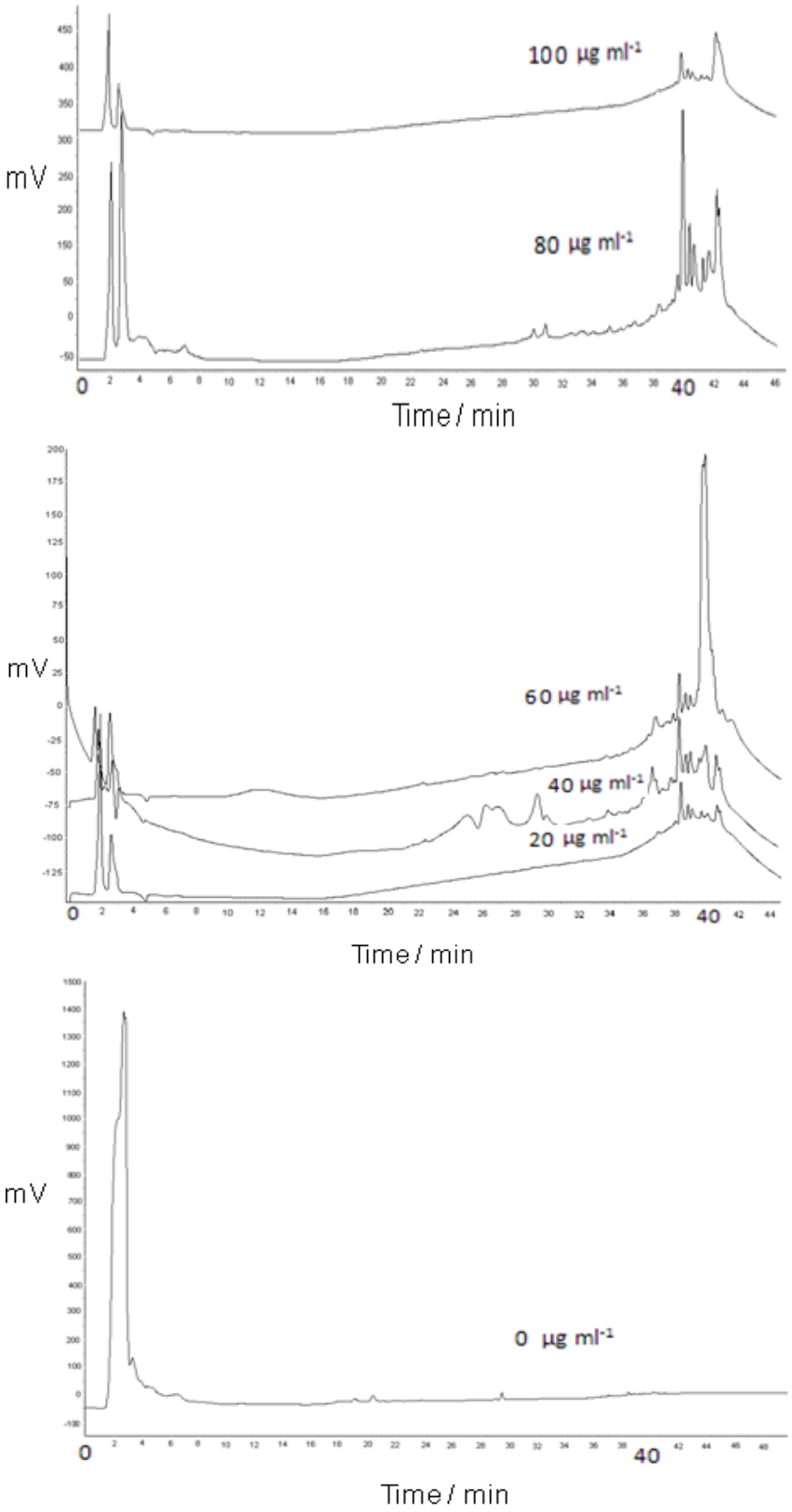

Figure 5-28. Lipochitooligosaccharide secreted (LCO) (at 39.8±0.8 minute) from *Bradyrhizobium* at different nanoparticle concentrations.

5.7.3 Effect of magnetite nanoparticle concentration on quantity acetylene reduction (an indicator of nitrogen fixation)

Analysis of variation (Table 5-85) shows the nanoparticles significantly increased nodule numbers per plant, nodule weight per plant and generated C_2H_4, (Figure 5-29). The results suggest that mainly increasing nitrogen genrated from nodules is due to increasing number of nodules per plant (Figure 5-29). Comparisons of the average number of nodule per plant shows the lowest average nodule numbers was obtained by control treatment and 20 µg $ml^{-1}$of the nanoparticles (Table 5-86), and the greatest average number of nodule was obtained by 60 µg $ml^{-1}$of the nanoparticles.

Table 5-85. Mean squares from analysis of variation of the generated C_2H_4 and nodule.

Source of variation	df	Mean squares					
		Average number of nodule / $plant^{-1}$	Average total nodules dry weight / mg $plant^{-1}$	Average dry weight of single nodule / mg^{-1}	Average generated C_2H_4 nmole h^{-1} (mg $nodules^{-1}$)	Average generated C_2H_4 nmole h^{-1} (mg $nodule^{-1}$)	Average generated C_2H_4 nmole h^{-1} (single $nodule^{-1}$)
Blocks	3	3077	6137	0.332	1976432	7.644	562
Nanoparticles	5	44146*	114327**	$0.407^{n.s.}$	3594980066**	2830**	31119**
Residual	15	11727	19687	0.751	3029393	31	2791

** *F* test indicates significance at $P < 0.01$; $^{n.s.}$ indicates a nonsignificant result.

The results also shows the average total nodules dry weight was not significant between control treatment and 20 µg $ml^{-1}$of the nanoparticles (Table 5-87), while the greatest average total nodules dry weight was obtained by 60 µg $ml^{-1}$of the nanoparticles, however its different mean with 80 µg $ml^{-1}$of the nanoparticles was not significant (Table 5-87). Comparisons of the greatest average generated C_2H_4 per nodule was obtained by 80 µg $ml^{-1}$of the nanoparticles (Tables 5-87, 88 and 89). The results also shows differences of the average generated C_2H_4 per nodule, between control treatment and 20, 40 and 60 µg ml^{-1} of the nanoparticles were not significant (Tables 5-87, 88 and 89).

It is conceivable that nanoparticles could damage plant tissue during their passage throught it, hence inducing ethylene release. This possibility does not appear to have been investigated.

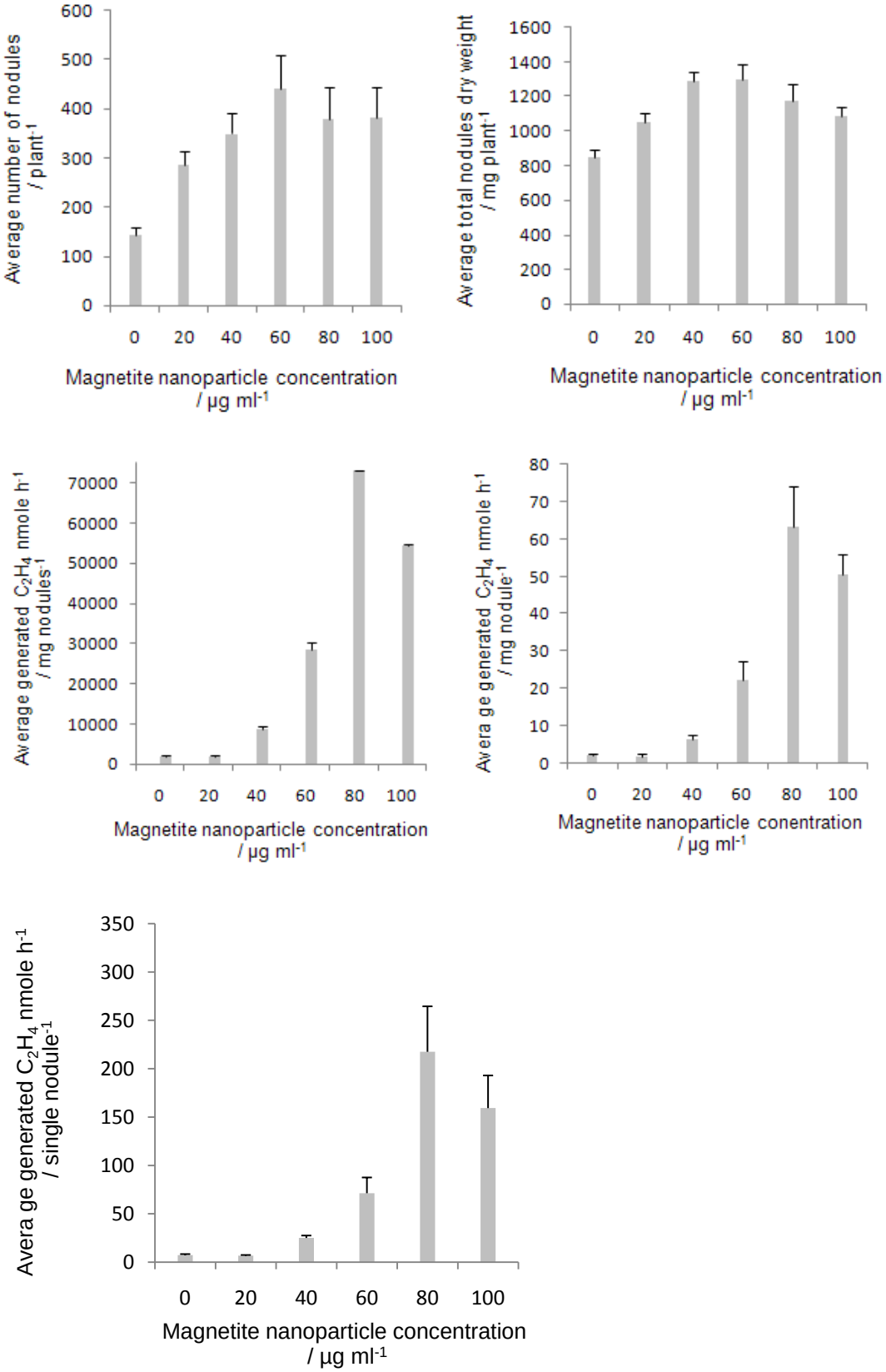

Figure 5-29. Response of (left upper) the average number of nodules per plant, (right upper) average total nodules dry weight per plant, (left centre) average generated C_2H_4 nmole h^{-1} per mg nodules, (right centre) average generated C_2H_4 nmole h^{-1} per mg nodule and (left bottom) average generated C_2H_4 nmole h^{-1} per single nodule under magnetite nanoparticle concentration effect. Error bars show standard error.

Table 5-86. Comparisons of the average number of nodules per plant under magnetite nanoparticle concentration effect.

Magnetite nanoparticle concentration / µg ml^{-1}	Average number of nodules / $plant^{-1}$	LSD value = 163.2
		Alpha = 0.05
0	169.3	B
20	287.3	AB
40	349.5	A
60	442.5	A
80	377.8	A
100	381	A

Table 5-87. Comparison of the average total nodules dry weight per plant under magnetite nanoparticle concentration effect.

Magnetite nanoparticle concentration / µg ml^{-1}	Average of total nodules dry weight / mg $plant^{-1}$	LSD value = 211.5	LSD value = 292.4
		Alpha = 0.05	Alpha = 0.01
0	853	D	b
20	1050	CD	ab
40	1293	AB	a
60	1300	A	a
80	1178	ABC	a
100	1085	BC	ab

Table 5-88. Comparison of the average generated C_2H_4 per mg nodules under magnetite nanoparticle concentration effect.

Magnetite nanoparticle concentration / µg ml^{-1}	Average of generated C_2H_4 nmole h^{-1} / mg $nodules^{-1}$	LSD value = 2623	LSD value = 3627
		Alpha = 0.05	Alpha = 0.01
0	1836	E	e
20	1932	E	e
40	8578	D	d
60	28560	C	c
80	73250	A	a
100	54540	B	b

Table 5-89. Comparison of the average generated C_2H_4 per mg nodule under magnetite nanoparticle concentration effect.

Magnetite nanoparticle concentration / µg ml^{-1}	Average of average generated C_2H_4 nmole h^{-1} / mg $nodule^{-1}$	LSD value = 8.405	LSD value = 11.62
		Alpha = 0.05	Alpha = 0.01
0	2.149	D	d
20	1.870	D	d
40	6.616	D	d
60	22.36	C	c
80	63.45	A	a
100	50.67	B	b

Table 5-90. Comparison of means of magnetite nanoparticle concentration effect on average generated C_2H_4 nmole h^{-1} per nodule.

Magnetite nanoparticle concentration / µg ml^{-1}	Average of average generated C_2H_4 nmole h^{-1} / single $nodule^{-1}$	LSD value = 79.63	LSD value = 110.1
		Alpha = 0.05	Alpha = 0.01
0	7.352	B	c
20	6.821	B	c
40	25.10	B	c
60	71.31	B	bc
80	217.5	A	a
100	159.3	A	ab

5.7.4 Effect of magnetite nanobiocomposite on quantity of acetylene reduction (an indicator of nitrogen fixation)

Table 5-91 shows generated C_2H_4, weight and number of nodules affected by nanobiocomposites. Figure 5-30 shows that C_2H_4 generated from a single nodule considerably increased in the presence of pure magnetite nanoparticles (50µg ml^{-1}). In addition the results suggest that an increasing number of nodules per plant could compensate the decreasing ability of generated C_2H_4 by a single nodule. Comparisons of the average number of nodules per plant shows the greatest average number of nodules was obtained with pure magnetite nanoparticle and soylecithin nanobiocomposite (Table 5-92). However, the difference between the average number of nodule in soylecithin and PVA nanobiocomposite effects was not significant (Table 5-92).The average total nodule dry weight per plant increased by the pure magnetite nanoparticle and the nanobiocomposites. The difference of the nodule's weight between methylcellulose and soylecithin nanobiocomposite was not significant (Table 5-93).

Table 5-91. Mean squares from analysis of variation of the generated C_2H_4 and nodule.

Source of variation	df	Mean squares					
		Average number of nodules / plant^{-1}	Average total nodules dry weight / mg plant^{-1}	Average single nodule dry weight / mg plant^{-1}	Average generated C_2H_4 nmole h^{-1} (mg nodules^{-1})	Average generated C_2H_4 nmole h^{-1} (mg nodule^{-1})	Average generated C_2H_4 nmole h^{-1} (single nodule^{-1})
Blocks	3	1083	3218	10.7	2318837	3.08	1120
Magnetite nanobiocomposites	4	6261**	77205**	26.4**	11890835**	5.06$^{n.s.}$	2761**
Residual	12	514.3	6352	2.6	1149401	2.62	315

** *F* test indicates significance at $P < 0.01$; $^{n.s.}$ indicates a nonsignificant result.

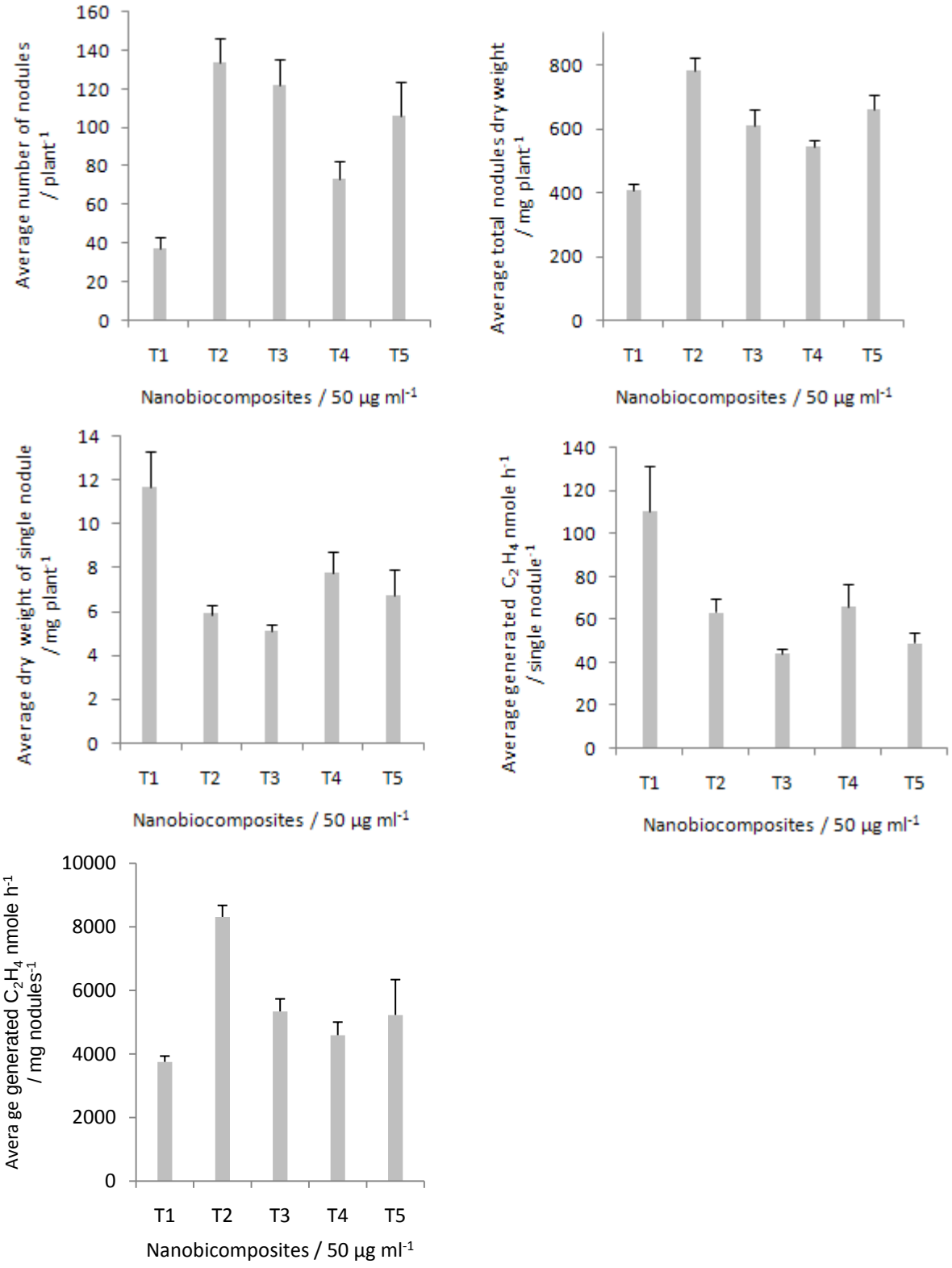

Figure 5-30. Response of (left upper) the average number of nodules per plant, (right upper) average total nodules dry weight per plant, (left centre) average dry weight of single nodule, (right centre) average generated C_2H_4 nmole h^{-1} per single $nodules^{-1}$ and (left bottom) average generated C_2H_4 nmole h^{-1} per mg nodules under nanobiocomposites effects; treatments (T_1= $B^{\dagger}$. without nanobiocomposite, T_2= B. + pure magnetite nanoparticle, T_3= B. + magnetite + soylecithin nanobiocomposite, T_4= B. + magnetite methylcellulose nanobiocomposite, T_5= B. + magnetite + polyvinyl alcohol (PVA). Error bars show standard error.

The greatest average generated C_2H_4 per nodules was obtained by pure magnetite nanoparticles, however, differences of the average generated C_2H_4 per nodules, between control treatment and nanobiocomposites were not significant (Table 5-94). Comparisons of the average weight of a single nodule show the greatest weight was obtained by control treatment, while the lowest weight was obtained by soylecithin nanobiocomposite; differences of the average between pure magnetite nanoparticle, PVA and methylcellulose nanobiocomposite were not significant (Table 5-95). The average generated C_2H_4 per a single nodule between control treatment and other treatments were significant; differences of the averages between nanobiocomposites and pure magnetite were not significant (Table 96).

Table 5-92. Comparison ofthe average number of nodules per plant under magnetite nanobiocomposites.

Magnetite nanobiocomposites concentration / 50 µg ml^{-1}	Average number of nodules / $plant^{-1}$	LSD value = 34.94 Alpha = 0.05	LSD value = 48.98 Alpha = 0.01
T_1= B†. without nanobiocomposite	37	C	c
T_2= B. + pure magnetite nanoparticle	135	A	a
T_3= B. + magnetite soylecithin nanobiocomposite	122	A	ab
T_4= B. + magnetite methylcellulose nanobiocomposite	74	B	bc
T_5= B. + magnetite PVA‡ nanocomposite	106	AB	ab

† *Bradyrhizobium japonicum*, ‡ polyvinyl alcohol.

Table 5-93. Comparison of the average generated C_2H_4 per mg nodules under magnetite nanobiocomposites.

Magnetite nanobiocomposites concentration / 50 µg ml^{-1}	Average generated C_2H_4 nmole h^{-1} (mg $nodules^{-1}$)	LSD value = 1652 Alpha = 0.05	LSD value = Alpha = 0.01
T_1= B†. without nanobiocomposite	3750	B	b
T_2= B. + pure magnetite nanoparticle	8313	A	a
T_3= B. + magnetite soylecithin nanobiocomposite	5337	B	b
T_4= B. + magnetite methylcellulose nanobiocomposite	4591	B	b
T_5= B. + magnetite PVA‡ nanocomposite	5225	B	b

† *Bradyrhizobium japonicum*, ‡ polyvinyl alcohol.

Table 5-94. Comparison of average total nodules dry weight per plant under magnetite nanobiocomposites.

Magnetite nanobiocomposites concentration / 50 µg ml^{-1}	Average total nodules dry weight / mg $plant^{-1}$	LSD value = 122.8 Alpha = 0.05	LSD value = 172.1 Alpha = 0.01
T_1= B†. without nanobiocomposite	405	C	c
T_2= B. + pure magnetite nanoparticle	780	A	a
T_3= B. + magnetite soylecithin nanobiocomposite	660	AB	ab
T_4= B. + magnetite methylcellulose nanobiocomposite	545	B	bc
T_5= B. + magnetite PVA‡ nanocomposite	613	B	ab

† *Bradyrhizobium japonicum*, ‡ polyvinyl alcohol.

Table 5-95. Comparison of the average dry weight of single nodule under magnetite nanobiocomposites.

Magnetite nanobiocomposites concentration / 50 µg ml^{-1}	Average dry weight of single nodule / mg^{-1}	LSD value = 2.507	LSD value = 3.515
		Alpha = 0.05	Alpha = 0.01
T_1= B†. without nanobiocomposite	11.67	A	a
T_2= B. + pure magnetite nanoparticle	5.90	BC	b
T_3= B. + magnetite soylecithin nanobiocomposite	5.09	C	b
T_4= B. + magnetite methylcellulose nanobiocomposite	7.76	B	b
T_5= B. + magnetite PVA‡ nanocomposite	6.74	BC	b

† *Bradyrhizobium japonicum*, ‡ polyvinyl alcohol.

Table 5-96. Comparison of the average generated C_2H_4 per single nodule under magnetite nanobiocomposites.

Magnetite nanobiocomposites concentration / 50 µg ml^{-1}	Average generated C_2H_4 nmole h^{-1} (single $nodule^{-1}$)	LSD value = 27.35	LSD value = 38.34
		Alpha = 0.05	Alpha = 0.01
T_1= B†. without nanobiocomposite	110.70	A	a
T_2= B. + pure magnetite nanoparticle	63.57	B	b
T_3= B. + magnetite soylecithin nanobiocomposite	44.23	B	b
T_4= B. + magnetite methylcellulose nanobiocomposite	66.25	B	b
T_5= B. + magnetite PVA‡ nanocomposite	49.11	B	b

† *Bradyrhizobium japonicum*, ‡ polyvinyl alcohol.

5.7.5 Effect of magnetite nanoparticle concentration on dry matter of vegetative components of soybean

Analysis of variation (Table 5-97) shows the average total dry matter of soybean plant and their vegetative components were not affected by nanoparticles after 60-70 days of planting (Figure 5-31), only branch numbers were affected by the nanoparticles. However, the average total dry matter, which includes nodule, root, leaf and stem, was increasing (Table 5-98), and as the measurement time was roughly near the end of vegetative growth (50-70 days before final plant growth), and also this variety was a member of determinate species (from 6-7 maturating groups) and predominately the maximum total dry matter will obtain after about 150-160 days; these are the likely reasons for the lack of effect of increasing generated C_2H_4 on the total dry matter or vegetative components. Comparisons of the average branches' numbers show that the differences of the averages between the control treatment and 40 or 60 µg $ml^{-1}$of the nanoparticle were significant, while comparing the averages between the control treatment and 20, 80 and 100 µg $ml^{-1}$of the nanoparticle were not significant (Table 5-99).

Table 5-97. Mean squares from analysis of variation of the components of vegetative growth under the nanoparticles.

Source of variation	df	Mean squares						
		Average total dry matter / g plant^{-1}	Average leaf dry weight / mg plant^{-1}	Average stem dry weight / g plant^{-1}	Average number of branches / plant^{-1}	Average plant height / cm	Average root dry weight / g plant^{-1}	Average number of nodes / plant^{-1}
Blocks	3	237	1.334	26.4	3	225	1.3	0.708
Nanoparticles	5	87$^{n.s.}$	0.548$^{n.s.}$	12.7$^{n.s.}$	5.2 *	132.6$^{n.s.}$	0.548$^{n.s.}$	0.542$^{n.s.}$
Residual	15	63	0.991	20.3	1.3	60.4	0.991	0.808

* *F* test indicates significance at $P < 0.05$; $^{n.s.}$ indicates a nonsignificant result.

Figure 5-31. Pictures of (top) the soybean plants after 20 days of planting and (bottom) roots (after 60-days) in a block under the nanoparticles effects, the treatments (T_0= without nanoparticles, T_{20}= 20 µg ml^{-1} of the nanoparticles, T_{40}= 40 µg ml^{-1} of the nanoparticles,T_{60}=60 µg ml^{-1} of the nanoparticles**,** T_{80}= 80 µg ml^{-1} of the nanoparticles , T_{100}= 100 µg ml^{-1} of the nanoparticles)

Table 5-98. Effect of the nanoparticles on average total dry matter of soybean plant after 60-70 of planting (the differences of average total dry matter between the treatments were not significant, see Table 5-97).

Magnetite nanoparticle concentration / µg ml^{-1}	Average total dry matter / g $plant^{-1}$
0	45
20	47
40	53
60	51
80	58
100	52

Table 5-99. Comparison of the average number of branches per plant under magnetite nanoparticle concentration effect.

Magnetite nanoparticle concentration / µg ml^{-1}	Average number of branches / $plant^{-1}$	LSD value =1.739	LSD value = 2.404
		Alpha = 0.05	Alpha = 0.01
0	11	A	a
20	10	AB	ab
40	8	B	b
60	9	B	b
80	11	A	ab
100	11	A	ab

5.7.6 Effect of magnetite nanobiocomposite on dry matter of vegetative components of soybean

Table 5-100 shows that average total dry matter of the soybean plant and their vegetative components were not significantly by nanocomposites; however, the dry root weight per plant was affected by nanocomposite treatments (Figure 5-32), and also the weight of total dry matter was increasing (Table 5-101), and as the measurement time was before the final plant growth, there was no observable effect of increasing generated C_2H_4 on the dry weight of the plant. The highest average difference was between the control treatment and pure mangnetite nanoparticle (Table 5-102). Comparisons of the average root dry weights show that differences of the averages between pure magnetite nanoparticle and PVA nanobiocomposite were not significant; in addition, differences of the averages between control treatment, soylecithin and methylcellulose nanobiocomposites were not significant (Table 5-100).

Table 5-100. Mean squares from analysis of variation of the components of vegetative growth in the presence of nanobiocomposites.

Source of variation	df	Mean squares						
		Average total dry matter / g plant^{-1}	Average leaf dry weight / mg plant^{-1}	Average stem dry weight / g plant^{-1}	Average number of branches / plant^{-1}	Average plant height / cm	Average root dry / g plant^{-1}	Average number of nodes / plant^{-1}
Blocks	3	2678	971	279	18	1542	17.2	42
Magnetite nanobiocomposites	4	60	13.5$^{n.s}$	3$^{n.s.}$	3.4$^{n.s.}$	119$^{n.s.}$	19.2**	1.2$^{n.s.}$
Residual	12	47	14.5	6.8	3.5	56	3.2	2.2

** *F* test indicates significance at $P < 0.01$; $^{n.s.}$ indicates a nonsignificant result.

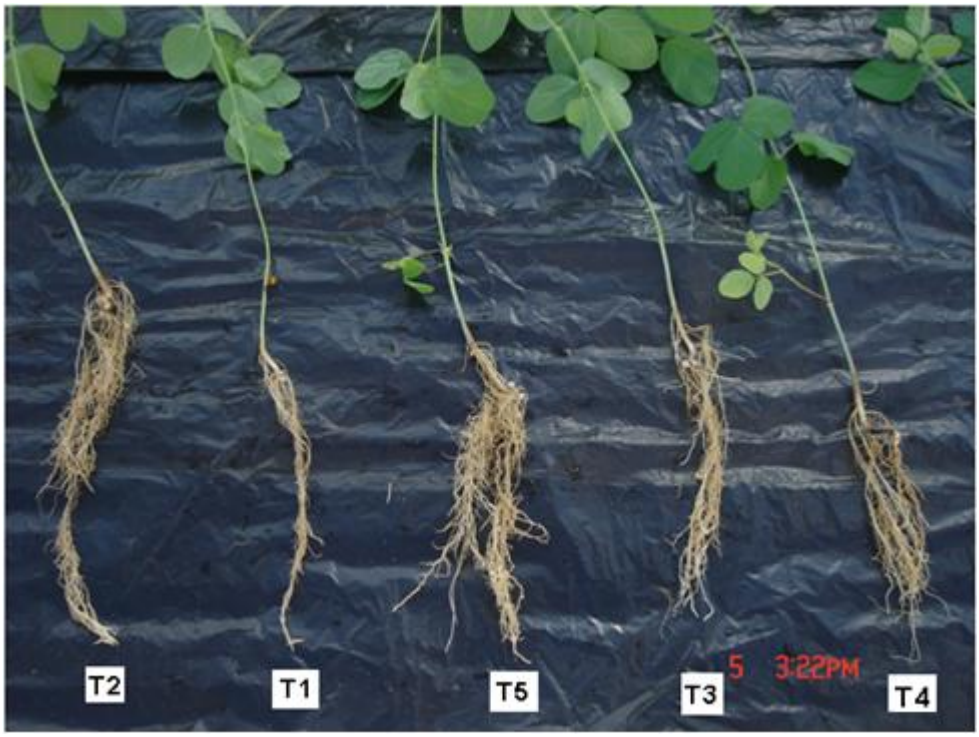

Figure 5-32. Pictures of the nanocomposites' treatments effect on the (top) soybean plants and (bottom) the roots (after 65 days of planting) in a block, the treatments (T_1= $B^{\dagger}$. without nanobiocomposite, T_2= B. + pure magnetite nanoparticle, T_3= B. + magnetite + soylecithin nanobiocomposite, T_4= B. + magnetite methylcellulose nanobiocomposite, T_5= B. + magnetite + polyvinyl alcohol (PVA).

Table 5-101. Effect of nanobiocomposites on average total dry matter of soybean plant after 60-70 days of planting (the differences of average total dry matter between the treatments were not significant, see Table 5-100).

Magnetite nanobiocomposites concentration / 50 µg ml^{-1}	Average total dry matter / g $plant^{-1}$
T_1= B†. without nanobiocomposite	25
T_2= B. + pure magnetite nanoparticle	36
T_3= B. + magnetite soylecithin nanobiocomposite	33
T_4= B. + magnetite methylcellulose nanobiocomposite	30
T_5= B. + magnetite PVA‡ nanocomposite	31

† *Bradyrhizobium japonicum*, ‡ polyvinyl alcohol.

Table 5-102. Comparison of the average root dry weight per plant under magnetite

Magnetite nanobiocomposites concentration / 50 µg ml^{-1}	Average root dry weight / g $plant^{-1}$	LSD value =2.745 Alpha = 0.05	LSD value =3.849 Alpha = 0.01
T_1= B†. without nanobiocomposite	5.9	B	c
T_2= B. + pure magnetite nanoparticle	10.7	A	a
T_3= B. + magnetite soylecithin nanobiocomposite	6.8	B	bc
T_4= B. + magnetite methylcellulose nanobiocomposite	6.4	B	bc
T_5= B. + magnetite PVA‡ nanocomposite	9.9	A	ab

nanobiocomposites effect.
† *Bradyrhizobium japonicum*, ‡ polyvinyl alcohol.

5.7.7 Discussion

Based on the results from the experiments described above, application of 40 and 60 µg ml^{-1} nanoparticles affect secreted genistein from roots and nodf caused enhanced symbiosis and symbiotic nitrogen fixation. In addition, magnetite nanocomposite (fabricated magnetite nanoparticles with soylecithin as a natural polymer) affects the weight and number of nodules.

Bradyrhizobium decorated with magnetite nanoparticles increased their survived better, and secreted an increased amount of nodf. These nodfs are the main inducer for exudation of genistein from roots. In fact this molecular dialogue between *Bradyrhizobium* cells and host roots acts as a cycle and that continues until the amount of the secreted nodf decreases by rapid death of bacterial cells. The nanoparticles preserve cell walls and simultaneously scavenge radical molecules in the niche of bacterial cells and regulate indirectly activity of secondary metabolites, which are important in the nodulation process as well as in fixing nitrogen. Hence increasing survival of cells on the surface of seed and roots caused increased induction of exudation of genistein, and consequently promotion of the nodulation process.

Generally the number of nodules and nodules weight was increased by application of the nanoparticles and the nanobiocomposites, but the response of C_2H_4 generated by a single nodule was different between them, which provides a reason for fabricating the different nanoparticles. One may presume that the ability of nanoparticles to reside on the surfaces of bacteria, seed and roots depends on physico-chemical properties of the nanoparticles. According to the results there is a direct relationship between an increasing number of nodules and generated C_2H_4. C_2H_4 generation from a single nodule after treatments with nanobiocomposites was lower than under control treatment.

The main purpose of using polymers in the fabrication of nanoparticles was to increase functional group concentration, hence increasing adhesion of the nanoparticles to the surface of the bacterial cells, and in turn increasing attachment of *Bradyrhizobium* cells as microsymbionts on the seed and roots, can facilitating the nodulation process. But based on treatments' comparisons, the differences of average generated C_2H_4 between control treatment and nanobiocomposites, were

not significant, the nanobiocomposites only increased number of nodules per plant. It is clear that nitrogen is one of the most essential nutrients for assimilation and production of photosynthates in the soybean. Growing and development of nodulation needs, however, carbohydrates. In fact the nodule is simultaneously a source to allocate materials of photosynthesis for growth and development of microsymbionts and nitrogenase enzyme and other requirements for the synthesis of proteins in bacteroids, and also is a sink to unload materials produced by photosynthesis from leaves to roots.

Nodules for their growing and development compete together to gain the photosynthates as well as with other parts of plant structure. Because of that, the control treatment (without the nanocomposite) led to more ability to generate C_2H_4 by a single nodule than other treatments; whereas C_2H_4 generated under other treatments promoted mainly via an increasing number of nodules per plant. In other words, the degree of competence in gaining photosynthates between the nodules caused decreased ability to generate C_2H_4.

However, literature suggests [27] the ferric uptake repressor (Fur) was secreted due to increasing concentration of iron in the niche of the bacteria; increasing concentration of the nanoparticles presumably induces ferric uptake repressor (Fur) proteins; capture of the nanoparticles by Fur leads to decrease benefit from the nanoparticles.

5.7.8 Conclusions

According to the results, decoration of *Bradyrhizobium* cells with 40 and 60 μg ml^{-1} of the magnetite nanoparticles could promote secretion of the nodf from *Bradyrhizobium* cells and genistein from host roots. Not only did the nanoparticles increase the number of nodules per plant, but also increased generated C_2H_4 by nodules. Note that the plants were grown in nutrient- and iron- free sand; hence the magnetite was the sole Fe source.

Magnetite nanobiocomposites (fabricated magnetite nanoparticles with soylecithin) could increase nodulation. Application of natural polymers like soylecithin in the fabrication of magnetite nanocomposite could increase functional groups for scavenging radical molecules and increase the ability of nanoparticles to reside on

the surface of bacterial cell, seed and roots.

Increasing nodulation in presence of the nanoparticles and nanobiocomposites could increase competence to allocate photosynthesis materials to nodules and roots, because of that nodules weight and roots per plant increased more significantly than other vegetative components

6 Final discussion

6.1 Quantity and characteristics of magnetite nanoparticles for symbiotic nitrogen fixation (SNF)

Nanoparticle concentration had an effective role in SNF: 40 and 60 µg ml^{-1} of magnetite nanoparticles with 10-20 nm mean diameter increased C_2H_4 generation, BGR, and cell viability. Fabricated iron particles in the nanoscale had four major benefits: (1) the free nanoparticles scavenge radical molecules; (2) residing on the surface of bacteria, seeds, and roots the nanoparticles acted as a protective layer against invading decomposers; (3) these nanoparticles compensate for iron deficiency and enhance oxygen availability to bacteria under extreme conditions; (4) the nanoparticles, with their huge surface area, have a buffering role and catalyse reactions tending to keep the pH neutral.

Due to the diminution of siderophore production the nanoparticles reduce energy expenditure under extreme conditions for bacterial cells, hence in the presence of the nanoparticles variables such as viability and C_2H_2 reduction as indicator of nitrogen fixation increased. The use of iron particles in the nanoscale to coat the bacterial cells increases their survival under extreme conditions and causes enhanced SNF and development of legume cultivations under environmental stress.

Nanobiocomposites increased the ability of the nanoparticles to scavenge toxic radical molecules and preserve water availability, enhancing survival of inocula on seed and roots. Nanoparticles residing in a matrix of natural polymers increased the number of functional groups around the nanoparticles, and then increased the scavenging capacity of the nanoparticles to protect of *Bradyrhizobium* cell.

The nanocomposites preserved cell wall integrity, humidity and availabel oxygen, promoting cell viability. Properties of natural polymers, such as high molecular weight and hydrophilicity in the fabrication of the magnetite nanobiocomposites could decrease decomposition of the bacterial cell wall. Although to date application of polymers in synthesis of magnetite nanoparticles has been used to control particle size, it is likely that this is the first time that nanobiocomposites have been used for enhancement of the BGR. The results of the FTIR and the XRD express that the beneficial changes (e.g., size and functional groups) of the magnetite nanoparticles in the nanocomposite mainly depends on the

kind of polymer, and its properties such as molecular weight, hydrophilicity and nontoxicity. Thus using of the nature polymers like soylecithin and/or methylcellulose could be more useful for promotion of beneficial nanoparticles, because positive properties such as hydrophilicity and high molecular weight are considerable in the natural polymers. In addition, functional groups of polymers in the nanobiocomposites likely caused to limit capturing nanoparticles by the ferric uptake repressor proteins.

Therefore redox properties of the nanoparticles, their concentration, molecular weight of the natural polymers and hydrophilicity have a considerable capacity to increase the BGR, cell viability, nodulation and quantity of generated nitrogen. However, properties of polymers are a key point in the beneficial changes of the nanoparticles which are affected under extreme conditions, as they might be able to change which reduce positive effect of the nanoparticles.

6.2 Bradyrhizobium growth rate (BGR) enhancement by magnetite nanoparticles

The BGR implies increasing cellular constituents, which can lead to increased size and population of microorganisms. Under nutrient limitation and waste accumulation in batch culture the BGR decreases and causes to earlier cessation of exponential growth, which then enters the stationary phase [37]. Growth process involves numerous anabolic (synthesis of cell constituents and metabolites) and catabolic (breakdown of cell constituents and metabolites) reactions [153]. Mostly environmental factors such as nutrients and water availability, pH, temperature, oxygen concentration, pressure, and radiation influence microbial growth process [194].

However, the results show that the nanoparticles maintained normal conditions for bacterial growth under extreme conditions, and as a result enhanced the BGR. In fact iron oxidation occurs biologically (iron—siderophores complex delivers the ferric to the bacterial cell, which is reduced ferrous as it is taken up), and under aerobic conditions, Fe^{2+} tends to oxidize to the Fe^{3+} at pH > 5 [153]. Reduced iron is an important source of energy for metabolic reactions and survival of *rhizobia* species. Under extreme pH, iron oxidation (Fe^{2+} to Fe^{3+}, e.g., in the form oxyhydroxides) cause to use oxygen and competes with the bilological reaction [153].

Assimilation requires the reduction of the ferric form (Fe^{3+}) for uptake and incorporation into cell constituents. This process is driven by released siderophores from *Bradyrhizobium* cells, which complex Fe^{3+} then delivers to the cell, which is reduced to ferrous form (Fe^{2+}) as it is taken up [153].

Likewise, for energy generation under limitation of oxygen dissimilatory iron reduction process or iron respiration, Fe^{3+} is used as a terminal electron acceptor for the producing energy. There are two strategies to generate energy due to association of the *Bradyrhizobium* cells and the nanoparticles. The first strategy is that the *Bradyrhizobium* cells make contact directly with iron oxide nanoparticles' surface, and then the iron nanoparticle reductase could be a membrane-bound enzyme, allowing direct access of the enzyme with the substrate. Second strategy is to utilize of released compounds from *Bradyrhizobium* cells during growth, which are contained of quinonelike electron shuttle. In fact these released endogenous electron shuttles can act as an intermediate in transferring electron from the cell to the iron oxide surface.

The physiological growth indices of *Bradyrhizobium* cell growth had a positive response to application of the nanoparticles, so that the MGT decreased approximately one hour under extreme conditions and/or the number of generation increased approximately from 18 times (control treatment) to 23 times (with 40 μg ml^{-1} of the nanoparticles) under the same conditions (e.g., see Figure 5-5). Magnetite nanoparticles have a pH buffering effect and they absorb alkaline bacterial secretions and react with them. This buffering prevents rapid pH fluctuation, which harm the bacteria.

6.3 Decreasing effect of extreme conditions on *Bradyrhizobium* growth rate (BGR) by the nanoparticles

According to the results decreasing cell viability as one of the most important physiological growth indices under extreme conditions such as salinity stress or low or high pH. The *Bradyrhizobium* cells showed that in presence of the nanoparticles had tolerance to salinity, acidity and alkaline pH stresses. Resistance of *rhizobia* species to extreme conditions changes under culture conditions, although previous researches illustrated the *Bradyrhizobium* has low resistance to salinity stress (see

section 2.1.3.2) [229]. However salt tolerance is dependent upon the pH value, temperature, carbon source and ionic species present in the growth medium [48,258]. Fragility of the *Bradyrhizobium* cells in absenc of the nanoparticles is possibly due to decreasing water availability and increasing osmotic pressure in medium, which limit normal interior metabolic reactions of the bacterial cell. Salinity reduces the external water potential, and this in turn will direct outlay of the cell's energy to be used for osmotic adjustment, for instance maintenance rather than growth and reproduction.

Therefore use of the nanoparticles may have increased viability via their redox potential and supply of availabel water and oxygen molecules on their surface, and ability to inactivate radical molecules. Likewise there is a possibility that these nanoparticles have an effect on the osmotic potential, or act as a specific solutes like glutamate or betaine which increase salt tolerance in the bacteria.

6.4 Decreasing rapid death of *B. japonicum* on seed by magnetite nanoparticles

The nanoparticles could protect *Bradyrhizobium* cells as inocula on the seed under ambient temperature (25°C, with population about 10^5 ml^{-1}) for 3-4 days or at low temperature (4°C, with population about 10^5 ml^{-1}) for 8-9 days.

Legumes seeds coat contain substances like tannic acid whose role is to protect seed against invading microorganisms that are decomposers in soil [3,57]. Generally inocula residing on the seed surface cause the induction of synthesis and exudation of toxin inhibitors like tannic acid from the seed coat. Initially inocula are introduced as invader and decomposer, but later other secreted compounds enter from the seed coat, like flavonoids, *Bradyrhizobium* cells are induced to excrete some polysaccharides molecules like the LPS and the EPS, which are able to cope with the effect of the toxin inhibitors, although numbers of *Bradyrhizobium* cells die during these biological reactions. There is a possibility the nanoparticles bonded with the toxin inhibitors lead to decreased rapid death of *Bradyrhizobium* cells present as inocula on the surface of the seed. Furthermore when the seed germinates and grows due to an increasing quantity of the secreted flavonoids from the roots, cell viability is increased, but it depends on a successful relationship between the bacterial strain and the host plant species.

However, survival of microsymbionts applied to the surface of seeds is poor due to desiccation [28,44,55,258]. Some previous researches showed that the nature of the suspending medium on the recovery of bacteria cells after dehydration is important in survival of the inocula in the dry state [44,258]. In other word growing and multiplication of bacterial cells affect normal conditions such as optimum pH, temperature, water availability, nutrients, and waste accumulation in the medium. Thus the nanoparticles, via a buffering effect, iron respiration, scavenging of radical molecules, conjugation with secreted disaccharides from bacteria like trehalose and/or polysaccharides and disaccharides in the seed coat promote hydrophilic properties of the bacterial cell wall and seeds.

6.5 Responses of lipochitooligosaccharide (LCO) and genistein as signal molecules due to magnetite nanoparticles

Secreted signal molecules such as LCO from *Bradyrhizobium* cells and genistein from soybean roots were affected magnetite nanoparticles. In fact the nanoparticles act as an inducer for the exudation of signal molecules of symbiosis partners and reinforce nodulation and symbiotic nitrogen fixation. The main reason for secretion of the nodf from *Bradyrhizobium* cells is induction of genetic agents of the bacterial cell by secreted genistein from host roots. Successful association of microsymbionts and host roots and continuing exudation of signal molecules between them mainly depend on the quantity of exudations and a later positive response between symbiosis partners. Thus one of the most important factors is survival and number of viable cells, which can increase the quantity of the secreted LCO. Likewise increasing the number of genistein molecules from root hair cells is affected by secreted nodfs from bacterial cells, and exudation of genistein increases by receiving more nodf. Hence, a crucial factor in the symbiotic nitrogen fixation is secretion of the nodf from *Bradyrhizobium* cells.

Microorganisms residing on the surface of roots generally lead to induction of the defence system of the plant, and usually plants via exudation of antioxidants prevent infection by pathogens like bacteria, fungi and/or viruses. Flavonoids have antioxidant activity and plants inhibit alien microorganisms via these exuded antioxidants. Therefore successful symbiosis between a microorganism and plant depends on the ability of secreted chemical compounds from the microorganism to

cope with invading antioxidants. Moreover, the following possible explanations seem worth considerating:

1- The nanoparticles increased viability and survival due to increased LCO.
2- The nanoparticles increase secretion of genistein from root.

Note that the plants were grown in nutrient- and iron- free sand; hence the magnetite was the sole Fe source.

6.6 Increasing nodulation by *B. japonicum* coated with magnetite nanoparticles

Nodulation processes were affected by *Bradyrhizobium* cells coated with the magnetite nanoparticles and magnetite nanobiocomposites. Ability of nodulation in a legume plant is regulated by genetic agents and environmental conditions [40]. Besides increasing number of nodules per plant regardless of the effect of nutrients, assimilation is due to penetration of at least one bacterial cell into the meristem root hair cell, which later proliferates effectively, increasing the number of bacterial cells in the bacteroid zone. The magnetite nanoparticles and magnetite nanobiocomposites increased the number of nodules and nodule weight per plant. Increasing the number of viable cells and decreasing the occurrence of rapid death of *Bradyrhizobium* cells on seed and roots, and also increasing excretion of signal molecules, eventually increased the possible successful penetration of the bacterial cells into the meristem of the root hair cells. Furthermore there is another possibility to increase attachment and penetration of the bacterial cells into root hair cell via increasing the volume of functional groups by fabrication of the nanoparticles with naturalpolymers like soylecithin, which not only increase the number of functional groups but also promote hydrophilic properties in order to preserve water availability. Moreover application of the nanoparticles and/or magnetite nanocomposite in order to coat the bacterial cell can enhance successful penetration of the bacterial cell into root cells, and the nodulation process.

Note that the plants were grown in nutrient- and iron- free sand; hence the magnetite was the sole Fe source.

6.7 Enhancement of SNF by magnetite nanoparticles and nanobiocomposites

The SNF enhancement depends on activated nodules and this activation depends on activity of the nitrogenase enzyme. It is recognized via change of color of the central zone of a nodule from white and light green to red. Application of the nanoparticles and the nanobiocomposites increased total generated C_2H_4 as an indicator of fixed nitrogen per plant. Response of the generated C_2H_4 (as indicator of nitrogen fixation) by a single nodule differs under application of the nanoparticles and the nanobiocomposites, so that the generated C_2H_4 by a single nodule under the control treatments is higher than with the nanobiocomposites, while generated C_2H_4 under nanobiocomposite treatment increased by increasing the number of nodules per plant. Thus using nanoparticles and nanobiocomposites promotes survival and successful penetration of microsymbionts into the root cell, and also promotes nodulation. These magnetite nanoparticles and nanobiocomposites remove the ROS and RNS, inducing more secreted signal molecules from the symbiotic partners. Via reinforcement of iron respiration under extreme conditions [153] they enhance the quantity of the nitrogen fixed by nodules. This enhancement is due to nitrogenase efficiency, because the increasing number of nodules per plant is evidence that the nanoparticles effectively regulate the oxygen requirement of the nodules (both in and out of nodule) by synthesis of metalloproteins like leghemogolobin which only act in the nodule. In addition, synthesis of nitrogenase, the major enzyme complex in the fixation of nitrogen, needs to have iron available, and also other proteins needed for symbiotic N_2 fixation are metalloproteins, like the nodule-specific cytochromes and/or other proteins containing iron for transferring electrons [266].

6.8 Total dry matter (vegetative yield) of soybean by the nanoparticle and nanobiocomposites

The quantity of vegetative components (leaves, stems, number of nodes and height) of soybean until starting the reproductive phase were not affected by application of the nanoparticles and the nanobiocomposites. However, the PVA nanobiocomposite, and also the 50 µg ml^{-1} of the nanoparticles, inceased the root dry weight compared to the other treatments but, in contrast to other experiments, the

pure magnetite nanoparticles at different concentrations had no significant effect on the dry root. Furthermore, the nanoparticles at some concentrations decreased the number of branches. Therefore, there is no certain reason to say that the nanoparticles and the nanobiocomposites have an additive effect on the vegetative yield of soybean.

Increasing the total dry matter of soybean plant is due to increasing products of photosynthesis (photosynthates). The photosynthesis process needs to receive sufficient elemental nitrogen as the most essential nutrient to increase the yield of soybean. Nitrogen requirement in the legumes is supplied mainly via symbiotic nitrogen fixation. Also distribution of photosynthates in primary plant growth are allocated more to roots, because the rate of root growth is double that of shoots in legume plants. Also supplying nitrogen from a symbiotic source is very important via formation of nodules and increase of the maximum number of nodules before ending vegetative growth and/or at least before starting the reproductive stages, until the plant can utilize reserved photosynthates, and then can increase the final economic yields (which are pods and/or seeds).

Among comparisons of the vegetative components under application of the nanoparticles and the nanobiocomposites, only the number of branches decreased at 40 and 60 $\mu g\ ml^{-1}$ of the magnetite nanoparticles, although the number of branches is regulated by genetic agents and crop management. But comparisons of the nodules weight and number of nodules per plant implies that probably decreasing number of branches is related to competion between the growth of roots and nodules with shoots, which resulted in a decrease of the number of branches.

However, the partitioning of dry matter between root and shoot is a heritable characteristic determined by the genotype of the plant. Ultimately the nanoparticles and the nanobiocomposites, via increasing cell viability, induce more secretion of the signal molecules from symbiosis partners, and the increasing number of nodules enhanced total generated C_2H_4 (as an indicator for fixed nitrogen) per plant and, hence, supplying nitrogen for growth and development of the soybean plant.

6.9 Conclusions

The overall main achievements of this study are as follows:

1. *B. japonicum* growth indices are affected by magnetite nanoparticles. The optimum concentrations were 40 and 60 µg ml^{-1} of nanoparticles.
2. Poor *B. japonicum* growth under pH extreme condition can be revived by adding magnetite nanoparticles. The optimum concentration was 40 µg $ml^{-1}$of the nanoparticles.
3. *B. japonicum* resistance under salinity stress increased by adding 40 and 60 µg ml^{-1} of magnetite nanoparticles.
4. Viability of *Bradyrhizobium japonicum* is favourably affected by magnetite nanoparticles. Although all concentrations of nanoparticles could increase cell viability during desiccation and at low temperature, at concentrations of 80 and 100 µg ml^{-1} the effects were almost the same as without nanoparticles.
5. Nodule factor (nodf) and genistein are affected by magnetite nanoparticles. The maximum secreted nodf from bacterial cells in liquid medium was obtained by 60 µg ml^{-1} of nanoparticles and genistein by 40 µg ml^{-1} of nanoparticles.
6. Generated C_2H_4 was affected by magnetite nanoparticles, implying that symbiotic nitrogen fixation (SNF) is affected by magnetite nanoparticles.The optimum concentrations were 80 and 100 µg $ml^{-1}$of nanoparticles.
7. The magnetite nanobiocomposites could not increase generated C_2H_4, thus the SNF could not increase in a single nodule.
8. The vegetative components of soybean were not affected by the nanoparticles.

Note that the plants were grown in nutrient- and iron- free sand; hence the magnetite was the sole Fe source.

7 Future work

7.1 Effect of nanoparticles on secretion of siderophores and ferric uptake repressor proteins from different strains of *Bradyrhizobium*

As the results showed, the secreted compounds from bacteria likely are the most important responses to environmental stresses. The nanoparticles' positive effects imply promotion of secretion of bacterial metabolites against stresses. Identification of secreted metabolites from bacteria under stress, and measurement of secreted polysaccharides, disaccharides, siderophores and ferric uptake repressor, when bacteria experience extreme conditions, are highly important in order to reveal the effect of nanoparticles on the resistance of bacteria. At present, there is no a certain evidence to reveal which compound is able to have effective association with nanoparticles. Thus, the next work will be researching to identify secreted compounds from the bacteria under the nanoparticles.

Different of *rhizobia* strains differ in their response to environmental stresses. If a species experienced saline soils, likely if it is used as inoculum, it is able to show more resistance to environmental stresses. However, there is insufficient evidence about the mechanism. Hence, comparisons of the stains in the presence of nanoparticles, is interesting to reveal the effect of nanoparticles on their responses to environmental stresses. In addition, the variation of genetical effects on resistance in the presence of nanoparticles it can be investigated.

As the results were obtained in the lab, they need to be tested in field. In order to precisely determine the effect of the nanoparticles and the coated bacteria with nanoparticles on grain yield at final growthof the plant, this research should carry out in the field to final stage of growth plant.

Furthermore, an investigation is needed to determine the efficiency, inocula in different soils and different conditions, of coating bacteria with nanoparticles, in order to elaborate likely positive effects of the nanoparticles.

The properties of nanoparticles change under different fabrication methods and hybridation with polymers. Polymers can change the properties in order to adapt nanoparticles for use in biotic systems and develop positive effects of nanoparticles for promoting and improving biological mechanisms. Furthermore, natural polymers are more biocompatible materials for coating nanoparticles. But, there is not sufficient evidence about effects of hybridation of natural polymers and nanoparticles on the secreted metabolites of bacteria under the extreme conditions. Therefore, investigation about the relation of properties of natural polymers and the nanoparticles may show which properties are more important in the change of resistance of bacteria under extreme conditions.

Moreover, there are main three steps to determine effects of the nanoparticles and coated *rhizobia* on the plant: i) in labratory, test responses of coating different strains of *rhizobia* with nanoparticles under extreme conditions, ii) in soil labratory, test the ability bacteria as microsymbionts in different soils and conditions, iii) in field, test the abiliy of the inocula in order to form nodules on the roots under different environmental conditions.

Besides, as C_2H_4 is only an indicator of nitrogen fixation, therefore after doing the experiments above, there is a need to measure nitrogen fixation by direct methods, including measuring (a) plant nitrogen accumulation or (b) the quantity of nitrogen fixation using $^{15}N_2$ methods.

7.2 Transfer of symbiotic nitrogen fixation to nonleguminous plants

To date some investigations mainly via transgenic science try to employ symbiotic nitrogen fixation in the nonleguminous plants like maize, wheat and/or rice, and so far their achievements could not overcome difficulties due to incompatible molecular dialogue between symbiosis partners. Generally, genetic modification is risky and it is unclear what would be the ecological impacts of genetic manipulations in bacteria or plant.

Properties of the nanoparticles such as scavenging, nutrient, energy generator, inducing exudation of the signal molecules between symbiosis partners, and

buffering effect under extreme conditions have opened new ways in order to develop and transfer symbiotic nitrogen fixation ability to nonleguminous plants. Incompatible exudations of nonleguminous root for inducing *rhizobia* cells, which discharge the LCOs is the most important problem for association of *rhizobia* strains with the roots of nonleguminous plants.

However results of this study suggest that the nanoparticles could preserve microsymbionts against secreted flavonoids from the roots of nonleguminous plants, which will act as antioxidants and are non-effective in symbiosis. In fact *rhizobia* coated with the magnetite nanoparticles induce effectively secretion of the LCOs and increase their quantity of secreted LCOs from *rhizobia* strains. This is a key point for defence against microsymbionts. Consequently, the coated *rhizobia* not only overcome secreted antioxidants from roots but also they infect effectively and form faster infection threads, and finally develop nodules and fix nitrogen.

This strategy of using the nanoparticles tries to diminish incompatible exudations of symbiosis partners (likely via their redox potential and inducing secretion of polysaccharides and disaccharides as a defence system against anitioxidants which are normaly secreted from plant root) in order to promte nodule formation and produce symbiotic nitrogen fixation.

7.3 Applications to related areas

7.3.1 Nano-fertilizers

Doubtless decreasing quantity of artificial fertilizers in farms is one of the most important efforts to prevent degradation of the environment, and also diminish the spreading of pollutants in the environment. Producing nanofertilizers at least could be an effective effort for decreasing consumption of fertilizers in farms.

It is possible to mention that the nanoparticles as fertilizers are a new type of toxic materials and they invade the ecosystem, because synthesis of materials in the nanoscale results in new properties of materials.

However, it is clear that generally fertilizer use efficiency on average in agricultural farms is about 50% [11], consequently the impact of fertilizer residues

would be volatilization to air, remaining as toxic elements in soil, leaching and entering ground water and/or all these events occurring together and leading to environmental degradation.

Otherwise all nutrients are absorbed in ionic form, and these ions should have a desirable dimension until they could pass through root cells. Hence these ions' dimensions are less than 1 micron meter, even probably less than 1 nanometer. However, changing size probably could produce undesirable properties for plant growth and should be investigated. Likewise export and import of a nutrient is carried by specific proteins in membrane of cells and cell walls of plant. Because of the importance of the surface to volume ratio engineered nanoparticles could be utilized in order to increase fertilizer use efficiency in farms. Furthermore, producing nano-fertilizers could be cost effective, and cause to decrease direct/indirect costs of producing agricultural products.

7.3.2 Nanoparticles for diagnostics

The magnetite nanoparticles could be used to recognize the ability of *rhizobia* strains to associate with host plant via inducing a quantity of secreted signal molecules of symbiosis partners, and even for discrimination against the other bacteria. Some bacteria cheat by infecting a plant root and only consume synthesised materials of the plant without producing fixed nitrogen. Also nanoparticles via their redox potential can induce different responses of physiological indices of bacteria growth resulting in new ways to diagnose resistance of bacteria against environmental stress.

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